# Recognition of microbial viability via TLR8 promotes innate and adaptive immunity

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All life is an experiment. The more experiments you make the better.

*Ralph Waldo Emerson, 1841*

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# Summary

Immune responses need to be tightly controlled in order to prevent unnecessary inflammatory tissue damage. It is therefore assumed that innate immune responses are scaled to the level of infectious threat posed by a given microbial encounter.

A recently described novel class of *pathogen associated molecular patterns* (PAMPs), termed *viability associated PAMPs* (*vita*-PAMPs), is exclusively present in live microorganisms and signals the presence of viable microbes and hence an increased level of infectious threat to the immune system. As a result, detection of *vita*-PAMPs elicits robust immune responses in murine phagocytes. The present study investigated innate immune recognition of bacterial viability in primary human *antigen presenting cells* (APCs) and its downstream effects on adaptive immunity. Human primary $CD14^{+}CD16^{-}$ (classical) monocytes were found to precisely distinguish between living and killed bacteria, independently of bacterial species, their replication capacity or virulence. Genome-wide transcriptome analysis revealed a selective regulation of a transcriptional core signature in response to viable bacteria, compared to heat-killed bacteria. Among the regulated genes, the inflammatory cytokines IL-12 and TNF were expressed nearly exclusively in response to living bacteria. The capacity to sense bacterial viability is not restricted to the murine and human immune system, as our study shows it is conserved across several evolutionarily distant species, such as swine and fish. Bacterial RNA was first identified as a *vita*-PAMP in mice. Supplementation of killed bacteria with bacterial RNA, or with synthetic ligands of *Toll-like receptor* (TLR) 7 and TLR8, but not with any other TLR ligands, fully restored TNF and IL-12 responses in human monocytes. Conversely, silencing the gene expression of TLR8 or of its signaling adaptor MyD88 in human and porcine monocytes abrogated viability-induced cytokine responses.

*T follicular helper* ($T_{FH}$) cells represent a specialized subset of CD4 helper T cells with a central role in the initiation of robust humoral responses. This study demonstrates that recognition of live bacteria, bacterial RNA, or synthetic TLR8 ligands renders human APCs potent inducers of $T_{FH}$ cell differentiation, whereas dead bacteria and other TLR agonists fail to do so. $T_{FH}$ differentiation in response to live bacteria is dependent on the recognition of RNA via TLR8 and the subsequent production of IL-12. Consequently, immunization of landrace pigs with a commercial live attenuated bacterial vaccine induced more robust $T_{FH}$ cell- and antibody responses compared to immunization with the heat-killed version of the same vaccine. In conclusion, we have identified TLR8 as the first known receptor for bacterial *vita*-PAMPs and revealed a critical role for TLR8 mediated 'viability sensing' in the induction of $T_{FH}$ cell and vaccine responses. These findings may have important implications for the development of novel vaccines and vaccine adjuvants.

# Zusammenfassung

Um unnötige Entzündung und Gewebeschäden zu vermeiden, werden antimikrobielle Immunantworten an den Grad der infektiösen Bedrohung angepasst. Eine kürzlich beschriebene neue Klasse *Pathogen-assoziierter molekularer Muster* (PAMPs), die als *Vitalitäts-assoziierte PAMPs* (*vita*-PAMPs) bezeichnet werden, ist ausschließlich in lebenden Mikroorganismen vorhanden. Sie signalisieren dem Immunsystem die Präsenz lebender Mikroben und zeigen damit eine erhöhte infektiöse Gefahr an. Die Detektion von *vita*-PAMPs löst in murinen Phagozyten verstärkte Immunantworten hervor.

Die vorliegende Studie untersuchte die Erkennung von bakterieller Vitalität durch humane *Antigen-präsentierenden Zellen* (APC), sowie die nachgeschalteten Effekte auf die adaptive Immunität. Humane $CD14^+$ $CD16^-$ (klassische) Monozyten unterscheiden sehr präzise zwischen lebenden und abgetöteten Bakterien, unabhängig von Bakterienarten, ihrer Replikationskapazität oder Virulenz. Eine genomweite Transkriptomanalyse zeigte eine selektive Regulation einer transkriptionellen Kernsignatur als Antwort auf lebensfähige Bakterien im Vergleich zu hitzegetöteten Bakterien. Unter den regulierten Genen wurden die inflammatorischen Zytokine IL-12 und TNF fast ausschließlich als Reaktion auf lebende Bakterien exprimiert. Die Fähigkeit, bakterielle Vitalität zu erkennen, ist nicht auf das murine und menschliche Immunsystem beschränkt. Die vorliegende Studie zeigt, dass diese Eigenschaft in evolutionär entfernte Spezies wie Schweine und Fische konserviert ist. Bakterielle RNA wurde zuerst in Mäusen als *vita*-PAMP identifiziert. Die Supplementierung von abgetöteten Bakterien mit bakterieller RNA oder mit synthetischen Liganden des *Toll-like-Rezeptors* (TLR) 7 und TLR8, jedoch nicht mit anderen TLR-Liganden, führte zu vollständig wiederhergestellten TNF- und IL-12-Antworten in humanen Monozyten. Umgekehrt hob die Unterdrückung der Genexpression von TLR8 oder seines signalgebenden Adaptors MyD88 in humanen und porcinen Monocyten vitalitätsinduzierte Zytokinregulation auf. *T-follikuläre Helferzellen* ($T_{FH}$) stellen eine spezialisierte Untergruppe von CD4-Helfer-T-Zellen dar, die eine zentrale Rolle bei der Initiierung von robusten humoralen Immunantworten spielen. Diese Studie zeigt, dass die Erkennung von lebenden Bakterien, bakterieller RNA oder synthetischen TLR8-Liganden durch menschliche APC zu einer robusten $T_{FH}$-Zelldifferenzierung führt, während tote Bakterien und andere TLR-Agonisten dies nicht tun. Die $T_{FH}$-Differenzierung als Reaktion auf lebende Bakterien ist abhängig von der Erkennung von bakterieller RNA über TLR8 und der anschließenden Produktion von IL-12. Folglich induzierte die Immunisierung von Schweinen mit einem kommerziellen attenuierten bakteriellen Lebendimpfstoff deutlich robustere $T_{FH}$-Zell- und Antikörperreaktionen als eine Immunisierung mit der hitzeabgetöteten Version des gleichen Impfstoffes. Zusammenfassend haben wir TLR8 als den ersten bekannten Rezeptor für bakterielle *vita*-PAMPs identifiziert und eine entscheidende Rolle für die TLR8-vermittelte 'Vitalitätserkennung' für die Induktion von $T_{FH}$ Zellen und Impfreaktionen gezeigt. Diese Ergebnisse könnten wichtige Implikationen für die Entwicklung neuer Impfstoffe und Impfadjuvantien haben.

# 1 INTRODUCTION

## 1.1 Innate immunity: receptors and responses

Innate and adaptive immunity have evolved as complementary systems to detect and clear microbial invaders such as viruses, bacteria and parasites.

While adaptive immunity is found only in vertebrates, the evolutionarily ancient innate immune system is a widespread defence system found in nearly all multicellular organisms including plants[1]. The dramatic advances in our understanding of the molecular mechanisms of the innate immune system have elucidated a role far from the traditional model of non-specific immunity to a more comprehensive role of critical regulator of the adaptive immune cellular and humoral responses[2]. Once an invading pathogen has breached the body's anatomical and physical barriers, phagocytes of the innate immune system represent the first line of defence. As such, it needs to elicit responses potent enough to ensure microbial clearance while avoiding the deleterious effects of extensive inflammation and tissue necrosis[3].

The specificity and activity of innate immune cells, such as professional phagocytes and *antigen presenting cells* (APCs), are primarily based on receptors with the ability to recognize molecular structures, or *patterns*, which are conserved across microbial lifeforms and, thus, signal the presence of pathogens within sterile tissues. Lacking the genetic recombination potential at the foundation of the specificity of the adaptive arm of the immune system, the innate arm relies on these germline-encoded receptors. First proposed by Charles Janeway in 1989[4], *pattern recognition receptors* (PRRs) were postulated to recognize microbial-specific ligands termed, in turn, *pathogen associated molecular patterns* (PAMPs)[a]. Many pathogens have evolved

[a] Given the non-pathogenic role of commensal bacteria, the usage of *microbe-associated molecular patterns* (MAMP) had been proposed instead.

strategies to escape detection by the immune system, however, the limited number of PRRs overcome this potential limitation by targeting microbial components fundamentally important for microbial integrity, viability or virulence and, as such, less prone to adaptive mutations[1]. Janeway's seminal theory on PRR was subsequently confirmed by Bruno Lemaitre and Jules Hoffmann, who showed that a receptor called *Toll* was instrumental in recognizing fungal infections in *Drosophila* flies[5]. Janeway and Ruslan Medzhitov then characterized the first mammalian PRR, *Toll-like receptor 4* (TLR4)[6], which was later identified by the group of Bruce Beutler as the cell surface receptor responsible for the recognition of *lipopolysaccharide* (LPS)[7], an invariant component of the outer membrane of most Gram-negative bacteria. Pattern recognition receptors represented the "missing molecular link" between the innate and the adaptive immune system. The discoveries of pattern recognition receptors and dendritic cells were later recognized with the Nobel Prize in medicine in 2011[8].

By definition, PAMPs are present in all pathogens and include molecular signatures that span from lipids to lipoproteins, from carbohydrates to nucleic acids. These components are recognized, more or less redundantly, by one or more PRR safeguarding pathogens recognition during different stages of infection and coping with diverse routes of pathogen invasion. In addition to microbial ligands, it is now well-accepted that PRRs can also sense endogenous ligands released from injured or dying cells (as a consequence of both infectious and non-infectious inflammation). These signals have been termed *damage-associated molecular patterns* (DAMPs)[9].

Engagement of PRRs by their respective ligands initiates a signaling cascade that usually culminates in the transcription and secretion of proinflammatory cytokines, chemokines, type I *interferons* (IFN) and other inflammatory mediators, as well as upregulation of co-stimulatory molecules on APCs including monocytes, macrophages, *dendritic cells* (DC), and polymorphonuclear leukocytes[10]. These molecules are fundamental in the induction of inflammatory responses, which will support the eradication of pathogens through the recruitment of other immune effector cells and via direct killing mediated by phagocytosis and other antimicrobial strategies (release of *reactive oxygen species* (ROS), extracellular traps etc.)[11, 12]. These early activation processes not only trigger an immediate antimicrobial host response, but they also initiate and orchestrate the activation of the adaptive arm of the immune system centred on antigen specific B and T lymphocytes[13]. The expression of *major histocompatibility complex* (MHC) protein and of costimulatory molecules on the surface of APCs following PRR activation is, indeed, essential in perpetuating and amplifying the host defenses[13, 14].

According to their structure and protein domain homology, PRRs are often grouped into five major classes. These families include: *Toll-like receptors* (TLRs), *C-type lectin receptors* (CLRs), *nucleotide-binding oligomerization domain* (NOD)-*like receptors* (NLRs), *retinoic acid-inducible gene I* (RIG-I)-*like family of receptors* (RLRs) and the *cytosolic DNA sensors* (CDSs). Alternatively, the aforementioned receptors can also be classified in two groups according to their cellular localization: membrane bound receptors (either on the cell surface or in the endosomal membrane compartment; i.e. TLR and CLR) and cytosolic receptors (CDS, RLR and NLR)[15].

## 1.2 Nucleic acid sensing receptors

Per definition, several PAMPs are present exclusively in microbes: these include components of the bacterial cell membrane and cell walls, such as lipopolysaccharide and peptidoglycan. Their recognition by PRRs guarantees selective differentiation between host (*self*) and microorganism (*non-self*), thereby limiting unregulated and erroneous activation of the immune system. Nonetheless, a subclass of PRRs can also be triggered by molecular structures, which are universally conserved and shared by all life forms, including the mammalian hosts. This class includes cytosolic and membrane bound receptors with a specificity for nucleic acids. While bacteria possess the CRISPR/Cas9 system to fight bacteriophages[16] and invertebrates rely mainly on RNA interference for their antiviral defence[17, 18], vertebrates have evolved specific receptors to detect invading nucleic acids triggering signaling cascades and the activation of genes involved in antiviral and antibacterial immune responses. Non-self nucleic acids, as hallmarks of infection, are discriminated from self-nucleic acids mainly on the basis of three criteria: (i) their persistence and local accumulation due to resistance to endogenous nucleases, which normally promptly degrade endogenous nucleic acids; (ii) their localization in subcellular compartments normally devoid of cellular nucleic acids, such as endosomes/phagosomes; (iii) the presence of secondary structures or the presence/absence of chemical modifications in the nucleic acids[19, 20]. PRRs involved in the detection of foreign nucleic acids comprise a heterogeneous group of cytosolic and membrane bound receptors including TLRs, involved in recognition of both DNA (TLR9) and RNA (TLR3, TLR7 and TLR8), the RNA-sensing RLRs (RIG-I and *melanoma differentiation associated gene 5*, MDA5), and DNA sensors *absent in melanoma 2* (AIM2) and *GMP-AMP synthase* (cGAS)[21].

## 1.3 Toll-like receptors

First identified in *Drosophila melanogaster* as a protein involved in the determination of dorsoventral polarity, the Toll protein was subsequently shown to also promote anti-fungal immune responses in the fruit fly[5]. This initial observation led to the aforementioned discovery

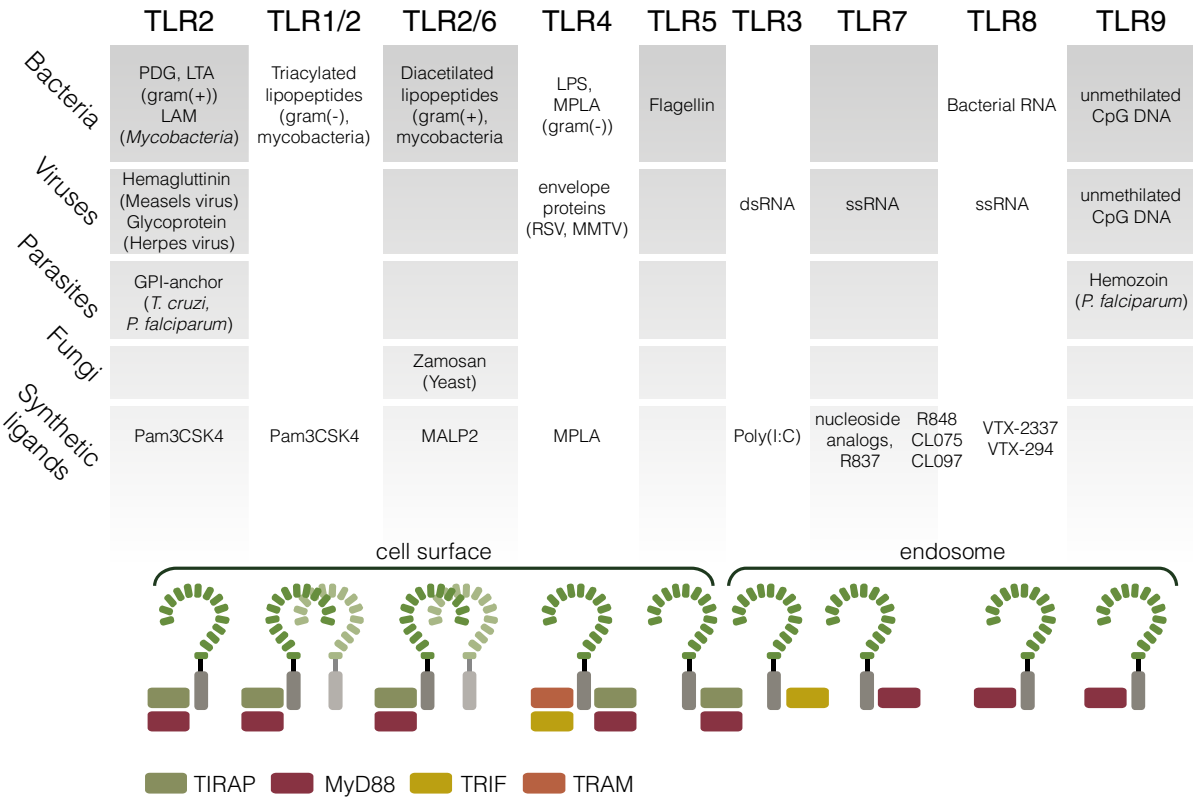


***Table 1. Summary of the ligands (either microbial or synthetic), structure and downstream signaling mediators of the human Toll-like receptors arranged according to their cellular localization***. For simplicity TLR10, whose ligand(s) are currently unknown, has been omitted. PDG: *peptidoglycan*; LTA: *lipoteichoic acid*, LPS: *lipopolysaccharide*; MPLA: *monophosphoryl lipid A*; RSV: *respiratory syncytial virus*; MMTV: *Mouse mammary tumor virus*; MALP2: *Macrophage-activating lipopeptide-2*; Poly(I:C): *Polyinosine-polycytidylic acid.*

of the first TLR homolog in mammals, TLR4[6]. Two decades after their discovery, TLRs likely remain the best characterized class of PRRs with generally detailed knowledge on their structure, ligand specificity ad downstream signaling pathways[22].

To date, ten functional TLRs have been identified in humans (TLR1 to TLR10), while twelve TLRs have been identified in mice. TLR11, TLR12 and TLR13 are exclusively present in mice (TLR11 is present in the human genome as a non-functional pseudogene[23]). Conversely, TLR10 has been lost in the mouse lineage, due to breakaway retroviral insertions[24]. Moreover, TLRs are present outside of the *Mammalia* class (including fish[25] and amphibians[26]), as well as in invertebrates[27], further underscoring the importance of this class of PRRs.

Despite their differences in cellular localization and ligand specificity (summarized in ***Table 1***), all known TLRs share a common molecular structure. TLRs are type I transmembrane integral glycoproteins composed of an N-terminal ectodomain containing a variable number of *leucine-rich repeats* (LRRs) responsible for ligand binding and receptor dimerization; a transmembrane single helix domain; and a C-terminal cytoplasmic *Toll-interleukin 1 (IL-1)-receptor* (TIR) domain which is essential for signal transduction upon ligand binding[28] and shares homology, hence its name, with the signaling domains of IL-1 receptor family members[22, 28].

The ectodomains of TLRs differ considerably in length, spanning from 550 to 800 amino acid residues, and face either the extracellular space (surface TLR) or the lumen of the endosomes (endosomal TLR) where they can recognize molecules released by invading pathogens such as microbial nucleic acids. Each TLR ectodomain contains between 19 to 25 LRRs, typically 22–29 residues long, which assemble into a characteristic α/β horseshoe solenoid configuration with a concave inner surface of parallel β strands, a convex surface formed by helices, β turns, and loops, and an internal hydrophobic core packed with leucine residues[28, 29]. The variability of LRRs between different TLRs is thought to be the central determinant of ligand specificity. The crystal structure of several TLRs in complex with their respective ligand has been determined and revealed how ligand binding occurs primarily in the lateral surface of the ectodomain bent structure[28, 30]. This region of the receptor, completely devoid of N-linked glycans, is accessible to the ligands and it is additionally involved in the dimerization of the receptor itself, which, upon ligand binding, produces the peculiar *"m"* shaped structure formed by two closely interacting ectodomains. While most TLRs function as homodimers, either preformed or assembled after ligand recognition, TLR2 forms heterodimers with TLR1 or TLR6, expanding in this way its ligand repertoire[22].

Following ligand binding, conformation changes in the receptor ectodomain. The C-termini (preceding to the transmembrane domain) are brought in close proximity activating the receptor[31]. This structural rearrangement in turn promotes the dimerization of the TIR domains on the cytoplasmic tail creating a scaffold for the binding of adaptor molecules necessary for the initiation of downstream signaling. Different adaptors are recruited via TIR-TIR domain interactions including *myeloid differentiation primary response gene 88* (MyD88), *TIR domain–containing adaptor–inducing IFN-β* (TRIF; also known as TICAM-1), *TIR-containing adaptor protein* (TIRAP), and *TRIF-related adaptor molecul*e (TRAM)[32]. TLR signaling can be broadly divided into a MyD88-dependent and a MyD88-independent pathway according to the primary adaptor recruited. MyD88 is universally used by all TLRs with the exception of TLR3, which binds solely to TRIF, whereas TLR4 can activate both the MyD88

and the TRIF dependent pathways[22]. Additionally, while MyD88 can interact directly with some TLRs, TIRAP is necessary for its recruitment downstream of TLR1, TLR2, TLR4 and TLR6[28, 33]. Conversely, TLR4 uses TRAM to mediate the interaction with TRIF. Other accessory molecules are involved the activation of some TLRs: CD14 and *LPS binding protein* (LBP), for example, are required for TLR4 response to LPS, while CD36 is believed to be a co-receptor for the TLR2/TLR6 hetrodimer[15].

## MyD88 and TRIF-dependent pathways

After its recruitment to the cytoplasmic side of TLRs, MyD88 sequentially engages with members of the *IL-1 receptor-associated kinase* (IRAK) family assembling into a helical structure containing six to eight MyD88 molecules[34]. Within this complex, the ubiquitin ligase *TNF-associated factor 6* (TRAF6) mediates the recruitment of the preassembled *TGF-beta activated kinase 1* (TAK1). The spatial regulation of this kinase complex mediates two different downstream events. At the membrane site, phosphorylation of the beta subunit of the *IκB kinase* (IKK) complex ultimately leads to the transcription factor *nuclear factor kappa-light-chain-enhancer of activated B cells* (NF-κB) translocation into the nucleus, via phosphorylation, and subsequent degradation, of its inhibitor, IκBα. NF-κB initiates the transcription of inflammatory cytokines including, for instance, IL-6 and IL-12p40[15, 22, 35]. Alternatively, after cytosolic translocation of the TRAF6-TAK1 complex, TAK1 activates a cascade of *mitogen activated protein kinases* (MAPK) including *extracellular signal-regulated kinase* (ERK), *p38* and, *c-Jun N-terminal kinase* (JNK). In turn, they trigger transcription factors, such as AP-1, also in this case leading to the transcription of proinflammatory cytokines, chemokines and factors promoting cell maturation[35].

Other MyD88-dependent signaling pathways have been characterized in specific cell populations. MyD88 can interact directly or indirectly, via members of the IRAK, TRAF and IKK families leading to activation of *Interferon regulatory factor 5* (IRF5) modulating the induction of IL-6 and IL12p40[36-38]. IRF1 also interacts with and is activated by MyD88 following TLR activation promoting the expression of IFN-β (most prominently downstream of TLR7 in murine cDCs[39]), *Inducible nitric oxide synthase* (iNOS), and IL-12p35 expression[40]. In *plasmacytoid dendritic cells* (pDC), a unique subset of DC known for abundant production of type I interferons, activation of IRF7, downstream of TLR7 and TLR9, induces transcription of Type I IFNs[41, 42]. IRF8 has also been implicated in TLR9-depended responses to CpG DNA, suggesting a redundant role in IFN- and cytokine responses[43].

Stimulation of TLR4 and TLR3, as mentioned, also triggers the TRIF-dependent pathway which, in macrophages and DC, leads to the production of proinflammatory cytokines and type I IFN via activation of NF-κB/MAP kinases and IRF3 respectively[22, 44]. TLR4 in particular, can initiate both the TIRAP-MyD88 pathway, when located at the plasma membrane, and the TRIF-dependent signaling, following dynamin-dependent endocytosis and trafficking to the endosomes[22]. TRIF forms a multiprotein signalling complex activating TAK1 in a process similar to theMyD88-dependent pathway. Phosphorylated IRF3 and IRF7 can translocate to the nucleus thereby initiating transcription of type I IFNs and *IFN stimulatory genes* (ISGs)[32, 35].

## TLR8

Out of the ten-known human TLRs, as mentioned, TLR3, 7, 8, and 9 are located in the endosomal compartment where they recognize nucleic acids. Precise compartmentalization is, in fact, one important factor to assure selective differentiation between host (self) and microorganism (non-self), since endogenous DNA and RNA are usually excluded from the endosomal compartment.

The ligand-binding ectodomain of TLR8, and the phylogenetically and structurally related TLR7, facing the internal lumen of the organelle are prone to recognize ligands of microbial origin entering the (early/late) endosome or phagosome after phagocytosis, or as a result of endosomal trafficking during the replication cycle of numerous viruses[45]. The encoding genes, in nearly all vertebrate species whose genomes have been annotated, are located in tandem on the same chromosome (the X chromosome in humans), and they most likely appeared as duplication of a single ancestral gene[46]. The persistence of both receptors, despite their similar structure, strongly suggests a selective evolutionary pressure in maintaining two distinct receptors[47].

In contrast to the extensive knowledge on the trafficking, activation, and ligand specificity of TLR7, until recently, TLR8 has received comparably little attention[48]. This discrepancy is mainly due to the lack of conservation between human TLR8 and its murine homologue. Murine TLR8 (mTLR8) was initially thought to be non-functional since $TLR7^{-/-}$ macrophages where unresponsive to R848[49] (also called Resiquimod; an imidazoquinoline, a dual ligand of human TLR7 and TLR8) and the same null phenotype was observed in HEK293 cells transiently transfected with mTLR8[50]. However, it was later reported that a combination of poly(dT) ODN and R848 was able to stimulate mTLR8 and induced the secretion of TNF in the same cell line and in primary murine *peripheral blood mononuclear cell* (PBMCs)[51]. An

additional report revealed that *vaccinia virus* (VACV), whose DNA genome contains poly(A)/T-rich regions, is a potent inducers of IFN-α in pDCs in a TLR8-dependent but TLR7-independent fashion[52]. Conversely, another study provided conflicting evidence on the activation of NF-κB and TNF in response to poly(A)/T oligonucletodes[53] highlighting the ongoing controversy and the need of further clarification on the expression and functionality of TLR8 in different murine cell populations and in response to microbial or artificial stimuli.

Notwithstanding the controversies surrounding TLR8 function in mice, significant advances have been made in recent years regarding the biology of human TLR8. These advances were mainly fostered by the description of the crystallographic structure of human TLR8 bound to its artificial chemical ligands[31], and later bound to its natural ligand, *single-stranded RNA* (ssRNA)[54]. TLR8 features a relatively large ectodomain consisting of 26 LRRs forming an almost complete circle, which brings the N- and C-terminal into close proximity. An inserted 30 amino acids long loop region (Z-loop) is located between LRR 14 and 15[55]. As with other endocytic TLRs, proteolytic processing in the endolysosome is thought to be necessary for its activation. TLR8, in particular, is cleaved within the Z-loop by a furin-like proprotein convertase and cathepsins in the early/late endosome[56]. The *endoplasmic reticulum* (ER)-resident multispan transmembrane protein UNC93B1 is indispensable for intracellular localization and activity of endosomal TLRs and has been shown to physically associate with TLR8 mediating its targeting to the early endosome[57], where further processing of the receptor takes place. After cleavage, the two resulting parts remain associated, and form the ligand recognition and dimerization surfaces. Unless processed, the insertion loop occupies the dimerization interface and prevents the assembly of the receptor active form since TLR8, unlike other TLRs, exists as a preformed dimer stabilized by extensive contacts between the ectodomains of the two protomers[30, 31]. Crystal structures in complex with synthetic agonists demonstrated that, upon ligand binding, the preformed dimer undergoes considerable reorganization to bring residues in the two carboxyl termini into close proximity (~30 Å from ~53 Å in the unbound dimer) promoting subsequent dimerization of the intracellular TIR domains, recruitment of adaptor complexes, and downstream signalling[31].

Additional structural studies revealed that TLR8 naturally recognizes two degradation products of ssRNA, as opposed to a full-length RNA molecule as previously thought. A uridine mono-nucleoside and a short purine containing oligonucleotide (such as UGG, UAA or UGA[58]) are bound at two distinct sites on the receptor surface. The single uridine uses the same site where small chemical ligands (such as CL097, a derivate of R848) are ligated, while the oligonucleotide is bound by a newly identified region on the concave surface of the horseshoe ectodomain.

While artificial ligands are able to activate TLR8 due a higher affinity, synergistic binding of the two ssRNA degradation products is necessary for activation under physiological conditions[54, 59].

Both human TLR7 and TLR8 share a general ligand specificity for ssRNA but nonetheless display significant differences in their ligation capability. TLR8 has been shown to recognize ssRNA viruses[60], synthetic ssRNA and also to respond to synthetic purine analogue imidazoquinoline derivatives such as R848[50] or CL075 (also called 3M-002)[61]. It has also been proposed that TLR8 (and also TLR7) are involved in the recognition of single stranded *Small interfering RNA* (siRNA), mediating their observed immunostimulatory effect[62, 63], while duplexed siRNA are unlikely to be sterically accommodated by the receptor[54].

Sequence specificity of ssRNA for TLR8 is still a matter of debate, but the presence of uridine seems to be a minimal requirement as shown by both infection and structural studies[54]. Single stranded RNA containing poly(U)- or GU-rich motives, such as ssRNA40 from the U5 region of HIV-1, can stimulate TLR8[64, 65]. Other studies instead suggested that AU-rich RNAs mediate human TLR8 activation, while GU-rich regions stimulate both TLR7 and TLR8 immune responses[66]. Therefore, it seems that TLR8 discrimination between self and non-self ssRNA is based on the abnormal endosomal localization, rather than sequence differences. During an infection, viruses can gain access to the endosomal compartment and their degradation can expose the viral genome facilitating recognition by TLR7 and/or TLR8. In this light, it is not surprising that recently also bacterial RNA has been described as a ligand of TLR8 in human monocytes upon infection with *Escherichia coli*[67], Gram-positive *Enterococcus feacalis*[68], *Streptococcus pyogenes*[69], *Staphylococcus aureus*[70], and in the spirochete *Borrelia burgdorferi*[71] (the bacterial agent of Lyme disease). Moreover, phagosomal delivery of *B. burgdorferi* purified RNA was shown to promote the secretion of TNF and IFN-β in an IRF7-dependent manner. Conversely, *S. aureus* induced secretion of IFN-β and IL-12 appears to be dependent on IRF5[70]. As a sensor of bacterial RNA, TLR8 could also provide another layer of self *vs* non-self discrimination. Evidence suggests that RNA which is scarce in modified nucleosides, as it is the case with bacterial RNA, stimulates human TLR8. Mammalian RNA, which incorporates copious nucleoside modifications (pseudouridine, $m^5C$, $s^2U$, $m^5U$ and $m^6A$), has only modest immunostimulatory potential[67]. Similarly, also mitochondrial RNA which, according to the endosymbiotic theory, resembles a molecule of its bacterial ancestor, was found to stimulate myeloid cells via TLR8[58, 67]. Supporting this hypothesis, bacteria have evolved a number of systems to evade cellular immune responses: naturally occurring 2'-O-ribose methylation of *Escherichia coli* $tRNA^{Tyr}$ at position 18, indeed, has been shown to inhibit human TLR8 activation[72, 73].

A further layer of tight regulation that can partially explain controversial findings on TLR8 activation patterns is the cell specific expression patterns in various human cells. TLR7 is mainly found in B cells and pDCs, whereas TLR8 is highly expressed in monocytes, *monocyte-derived dendritic cells* (Mo-DCs), macrophages and *myeloid DCs* (mDCs), while its expression in pDCs is rather modest[61, 63, 74-76].

## 1.4 RNA sensing in the cytosol

As pointed out above, in addition to the TLRs, several other receptors are involved in the recognition of nucleic acids in the cell cytosol. In particular, RIG-I, the closely related family members MDA5 and *Laboratory of genetics and physiology* (LGP2) have been identified as sensors of viral RNA. More recently, RIG-I was also implicated in the recognition of bacterial RNA derived from *Listeria monocytogenes*[77]. The three RLRs share a common, highly conserved domain architecture composed of a central DExD/H helicase domain with ATPase activity, connected via a flexible pincer domain to a C-terminal section involved in the ligand specificity. RIG-I and MDA5 both possess an additional duplicate tandem N-terminal *Caspase activation and recruitment domain* (CARD) which mediates singling toward their common downstream adaptor protein *mitochondrial antiviral-signaling protein* (MAVS)[78, 79]. LGP2, on the other hand, lacks a CARD domain and, despite being able to recognize RNA, it is thought to be mainly involved in positive regulation of MDA5[20].

Several studies have elucidated the ligand specificity of MDA5 and RIG-I receptors. RIG-I preferentially binds to short blunt end *double-stranded RNA* (dsRNA; around 300bp long) and ssRNA harbouring a 5´triphospate group (5-ppp), a structure that is lacking in host endogenous RNA[80]. The presence of this chemical moiety is indicative of RNA of bacterial or viral origin being actively synthetized within the cytosols. Endogenous RNA generated in the nucleus, on the other side, undergoes further processing in the likes of backbone modifications and 5-ppp-linked methylguanosine ($_{m7}$G) cap, which abolishes the RIG-I immunostimulatory potential[19, 81]. The precise nature of the ligands sensed by MDA5 have remained more elusive, however, several studies suggest that it recognizes longer dsRNA (>300bp), as well has web-like mixed structure generated by high-molecular-weight RNA[78, 82].

In both instances, detection of the respective ligands leads to the ATP-dependent oligomerization of RIG-I and MDA5 in a head-to-tail arrangement exposing 2xCARD tetramers to recruit and activate the adaptor MAVS anchored in the outer membrane of mitochondria and peroxisomes[19, 83]. Triggered prion-like polymerization of MAVS proteins

ultimately results in the activation of downstream signaling pathway and the activation of transcription factors IRF1, IRF3, IRF7, and NF-κB inducing the expression of type I interferons and proinflammatory cytokines respectively[84, 85].

The mitochondrial adaptor protein MAVS has also been implicated in *NOD-, LRR and pyrin domain containing 3* (NLRP3) inflammasome activation, resulting in the secretion of mature IL-1β[86]. Inflammasomes are large multiprotein complexes, which assemble in the cytosol in response to various exogenous or endogenous stimuli and, therefore, play a central role in the innate immune responses to invading pathogens[87]. These large multimeric structures share a multipart architecture centred around a sensor protein which defines the class of the corresponding final complex. The sensors include members of the NOD like receptors (NLR) family, such as in the NLRP3 inflammasome, the *double-stranded DNA* (dsDNA) sensor AIM2 and the recently characterized pyrin inflammasome[88]. Upon detection of the activating stimulus, the sensors typically oligomerize and trigger the nucleation of self-propagating, prion-like assemblies of the adaptor *apoptosis-associated speck-like protein containing a caspase activation and recruitment domain* (ASC)[87, 89]. This large micrometre-sized structure, termed ASC-speck or *pyroptosome*, serves as a scaffold supporting docking of the cysteine protease procaspase-1 which is activated after proximity induced autocatalytic cleavage. The active subunits p10 and p20 are, in turn, able to proteolytically process the pro-forms of the inflammatory cytokines, IL-1β and IL-18, into their biologically active forms. Moreover inflammasome assembly in response to microbial or other danger signals, induces pyroptosis, an inflammatory form of programmed cell death[89], via cleavage of Gasdermin D[90], which subsequently inserts into the plasma membrane to form a pore[91].

Of the different known or putative inflammasomes, the NLRP3 inflammasome was the first one to be identified and, it is one of the best studied. The ever growing list of triggers of the canonical NLRP3 inflammasome spans from ATP to nigericin, from bacterial pore-forming toxins to cell wall components, from the vaccine adjuvant alum to organic or inorganic crystals and asbestos fibers[92]. Rather than interacting directly with this multitude of potential activators, the NLRP3 inflammasome is believed to sense common secondary cellular events induced by these triggers. Although the precise nature of these events is still controversial, consensus is building around potassium efflux as the main trigger mediating canonical NLRP3 activation[93]. Nucleic acids are also listed among the activators of the NLPR3 inflammasome, including synthetic dsRNA[94] and viral RNA[95, 96]. Additionally, the NLRP3 inflammasome has been linked to the recognition of cytosolic bacterial RNA of both Gram-positive and Gram-negative bacteria[97-100]. Interestingly, while only prokaryotic messenger RNA (mRNA) is able to activate the NLRP3 inflammasome

in murine innate immune cells, the human homologue in myeloid cells seems to be responsive to all bacterially derived RNAs tested (mRNA, transfer RNA or tRNA, and ribosomal RNA or rRNAs)[99, 101]. Moreover while 5'-triphosophate moieties, secondary and double stranded structures, as described, trigger RIG-I activation in the cytosol, the assembly of a functional inflammasome seems to be independent from these modifications[98, 99, 101].

The mechanism by which bacterial RNA gains access to the cytosol after productive infection and/or phagosomal degradation of ingested bacteria is unclear, however it was suggested that naturally occurring phagosomal leakage may release microbial RNA into the cytsol[99]. In particular, during Gram-negative bacterial infection this event leads to NLRP3 inflammasome activation, which is licensed in a TLR4-TRIF-IFN-β axis-dependent manner[102]. The exact mechanism by which NLRP3 senses, directly or indirectly, microbial RNA remains to be fully elucidated and both direct binding or recognition via the RNA helicase DHX33 have been proposed[100, 103].

## 1.5 Bacterial 'viability sensing' and vita-PAMPs

Recent observations support a critical role of a novel class of PAMPs associated with the viability status of encountered microbes and thus termed *viability-associated PAMPs* (*vita*-PAMPs)[3, 99].

*Vita*-PAMPs form an intriguing new class of PAMPs, the detection of which signifies bacterial viability to the immune system, thereby guiding the strength of both the innate and the adaptive downstream responses[3]. The central dogma stating that innate immunity primarily relies on self *vs* non-self discrimination it is not sufficient to explain the inherent ability of the mammalian immune system to make well-measured decisions based on subtler distinctions such as the discrimination between commensal and pathogenic microorganisms, or living and dead bacteria[104]. This extraordinary capability has important implications in the generation of appropriate immune responses and the avoidance of overshooting reactions. Most intriguingly, this phenomenon may also explain the inherent superiority of live attenuated vaccines over their dead counterparts.

Sander *et al.* in 2011 demonstrated that murine APCs (either *bone marrow derived macrophages* or BMDMs, peritoneal macrophages or dendritic cells) are indeed able to discriminate live from dead bacteria, independently of bacterial species, pathogenicity, or replication. Recognition of viable bacteria leads to the assembly of the NLRP3 inflammasome and the release of IL-1β, as well IFN-β. Only viable but not heat-killed bacteria induce

inflammasome activation, cleavage of pro-caspase-1 and pyroptosis. Proinflammatory cytokines IL-6 and TNF are induced in comparable amounts by both live and dead bacteria. The TLR adaptor protein TRIF was found to have a central role in regulating the NLRP3 inflammasome as *Trif*$^{-/-}$ cells fail to induce pro-caspase 1 cleavage and IL-1β secretion in response to live bacteria. This TRIF dependent phenotype was independent of transcriptional control of NLRP3 or pro-IL-1β. NLRP3, Caspase 1, the adaptor ASC, and the transcription factor IRF3 are all essential for the cellular responses to bacterial viability. The same pattern of TRIF dependent Il-1β secretion was also observed *in vivo* after intraperitoneal injection with viable *E. coli*[99].

Bacterial messenger RNA, in particular, was recognized as the first member of the *vita*-PAMP class of microbial stimuli. RNA is rapidly lost following bacterial killing and its presence correlates with the ability to activate immune cells. Purified bacterial mRNA, indeed, when combined with heat-killed bacteria restores their ability to induce pyroptosis, IFN-β and IL-1β production in a sequence independent manner. Under physiological conditions, RNA can – in theory - access the cytosol, and thus the inflammasome, due to an intrinsic low-level leakage of phagolysosomes[99]. Additionally, *vita*-PAMPs impact ensuing adaptive immune responses in mice, as animals vaccinated with live bacteria, or a combination of heat-killed bacteria and purified bacterial RNA, mount more robust antibody responses.

Beyond bacterial RNA only another *vita*-PAMP has been characterized to date: *cyclic-di-adenosine-monophosphate* (c-di-AMP) produced by live Gram-positive bacteria which induces the STING-dependent secretion of type-I interferon.

The process of bacterial 'viability recognition' in human APCs, and its impact on subsequent adaptive immune responses remained unknown and formed the objective of the present study.

Deconstructing the process of 'viability recognition' has profound significance in improving our understanding of the early events, or *check points*[3] of innate immunity, which enable precise evaluation of the infectious treat, thus ensuring appropriately tailored immune responses. Moreover, as mentioned, such knowledge would have important clinical impact, as it might help explain the notoriously higher activity of live attenuated vaccines, compared to inanimate subunit vaccines[105, 106]. Innate recognition of microbial nucleic acids, and pre-eminently RNA, could represent the culprit behind the success of live vaccines[3, 39, 99]. Therefore, precise definition of the mechanisms of 'viability sensing' in human APCs could provide essential clues for the rational design of efficient vaccines and vaccine adjuvants[107].

## 1.6 The adaptive immune response and its control by innate immunity

The recognition receptors and effector proteins of the innate immune system are generally germ-line encoded and expressed on an enormous variety of different cell subtypes. This ensures, as mentioned, a rapid and efficient recognition of microbial invaders[14]. However, the virtually unlimited variability of microbial antigens and the continuous reciprocal competitive pressure on the immune system and on bacterial and viral pathogens for survival[108], has driven the evolution of an adaptive immune system in all jawed vertabrates[4]. The central receptors of the adaptive immune response, namely the *T cell receptor* (TCR) and the *immunoglobulins* (Ig or *B cell receptor*, BCR) are generated via a process of somatic rearrangements and hypermutations of a large array of germline encoded gene segments. This process, and the following clonal selection of a functional receptor repertoire, allows for the generation of a vast amount of different receptors, with a sheer unlimited ligand specificity. These receptors are expressed on the surface of the central effectors cells of the adaptive immune system: the T and B lymphocytes[1].

Along with increased specificity, a second key feature of the adaptive immune system is its capability of maintaining a so-called *immunological memory*, based on long-lived cells which are rapidly reactivated after antigenic re-encounter, allowing for an effective and quick stimulation of memory B and T cells even decades after first antigen contact. This extraordinary ability forms the basis of vaccination[109, 110].

### T Lymphocytes

T cells develop in the thymus, where lymphoid progenitors, egressing from the bone marrow, expand in number and undergo further differentiation[1]. TCRs expressed on the surface of T cells are heterodimers composed of either αβ (95%) or γδ (5%) polypeptide chains each containing a variable region and a constant region proximal to the cell membrane. Tightly associated with the TCR is the invariant CD3 accessory multimeric protein complex, a defining feature of the T cell linage[1, 111]. While the TCR is responsible for engagement with the antigen-MHC complex (via its variable extracellular region), the CD3 complex transduces and amplifies the phosphorylation-mediated intracellular signals necessary for activation of the T cell upon antigen recognition. The massive repertoire of antigen-specific TCR αβ is generated, as mentioned, through an articulated process of genomic rearrangement between gene

segmnents[112]. Each random resulting rearranged region dictates the final amino acid composition of the receptor site devoted to antigen-MHC-recognition[1, 14].

T cells also express TCR co-receptors CD4 and CD8, which assist the TCR activity via interactions with constant domains of MHC class I and II, respectively, thus providing the MHC class restriction characteristic of $CD4^+$ and $CD8^+$ T cells. $CD8^+$ T cells possess cytotoxic activity against cells infected with intracellular pathogens or cancer cells expressing neo-antigens. $CD4^+$ cells, on the other side, are termed *helper cells* and are central in the activation of various arms of the immune response, in particular B cell based humoral immune responses[113]. After selection in the thymus, naïve $CD4^+$ or $CD8^+$ T cells exit into the circulation reaching secondary lymphoid organs including lymph nodes, the spleen and the mucosa-associated lymphoid tissues.

Both $CD4^+$ and $CD8^+$ cells TCRs must interact with antigenic peptides presented by MHC for them to become fully activated. Specifically, $CD4^+$ cells engage antigenic peptides presented in the context of MHC class II expressed on the surface of immune cells such as monocytes, DC, macrophages, or B cells, thus generally termed professional antigen presenting cells. These highly specialized phagocytic cells, patrol blood and peripheral tissues, while some of them reside within secondary lymphoid organs, sampling exogenous antigens and microbes, which they engulf by means of phagocytosis or endocytosis. Microbial encounters induce the maturation of APCs, which is generally mediated by the engagement of germline-encoded PRRs, leading to enhanced surface expression of MHC class II and co-stimulatory molecules and the secretion of cytokines and chemokines[1, 3], essential for downstream adaptive immune responses[2, 114]. Ingested exogenous proteins are proteolytically processed into short 15 to 24 amino acid long antigens which are then assembled into nascent MHC complexes and are translocated to the cell surface[115]. Following their activation, antigen loaded APCs migrate from peripheral infection sites to the draining lymph node where they can interact with antigen-specific resting $CD4^+$ (or $CD8^+$) T cells and promote their maturation into effector cells. The interaction between TCR and the antigenic peptides presented on MHC molecules provides the *first* signal required for T cell activation. This interaction is strengthened by the simultaneous binding of CD3 as well as of CD8 or CD4 molecules to non-polymorphic regions of MHC class I or MHC class II respectively forming an extensive region of cell-to-cell contact called immunological synapse[116]. A *second* fundamental signal is delivered by co-stimulatory molecules expressed on the surface of activated APC. These include CD80 (or B7.1) and CD86 (or B7.2) which engage with CD28 on the cognate T cell. Finally, a *third* signal comes from cytokines secreted by APCs at the site of T cell activation and whose nature, combination and balance influences the further differentiation of T cells[117, 118]. The three signals combined initiate an intracellular signalling

cascade that culminates in the coordinated activation of several transcription factors responsible for the activation of genes which promote proliferation and determine the phenotypic and functional outcome of the mature effector T cell[114, 119, 120].

## T-helper cell differentiation

$CD4^+$ T cells, as mentioned, are fundamental components of the adaptive immune response and their helper function is necessary for the generation of high affinity antibody responses and for the formation of long-lived plasma cells, memory B cells, as well as long-lived memory $CD8^+$ T cells[121, 122]. Moreover $CD4^+$ cells have important roles in orchestrating innate immune responses[1, 123].

Once a naïve T cell is primed by signals received from APCs, following proliferation[124], the cytokine milieu created by innate immune cells instructs differentiation into a particular $CD4^+$ effector cell subset each defined by lineage-specific transcription factors and often named after the key cytokines expressed by the respective helper cell subset[114]. The family of human T helper ($T_H$) subsets includes T helper 1 ($T_H1$), and T helper 2 ($T_H2$) cell, the earliest subsets to be described, T helper 17 ($T_H17$), T helper 9 ($T_H9$), T helper 22 ($T_H22$), regulatory T cells ($T_{reg}$) and *T follicular helper* ($T_{FH}$) cells. Each population is characterized by distinct features and specialized functions, which have most likely evolved to counteract specific classes of pathogenic challenges. The strict separation between the different T helper cell subsets has, however, been revisited in recent years. Given the considerable degree of plasticity, the different subsets may rather reflect transient polarization states than terminal differentiation fates of T cells. Much like the cells themselves, this area is still a dynamic field of investigation. Within the conceptual framework of the 'three-signal-theory' (signal 1: antigen, signal 2: co-stimulation, signal 3: cytokines) required for lymphocyte activation, T cells can integrate a complex combination of environmental cues, including the cytokine milieu, the particular features of the APCs and those of the presented antingen[125-127]. Other factors have also been reported to play a role in the development of effector T cells including the strength and persistence of the of signals via T cell receptor[128], the intracellular epigenetic landscape and microRNA activity[120, 129], metabolic states, the availability of nutrients and oxygen, as well as extracellular salt concentrations[130]. Overall, a complex combination of extracellular and intracellular cues is integrated to generate optimally adapted T cell responses.

The pathways skewing naïve T cells towards the $T_H1$ lineage are largely conserved in mice and humans[114]. The cytokine IL-12 secreted by APCs in concert with interferon-γ (IFN-γ) produced

by both *natural killer* (NK) cells and T cells are generally thought to strongly induce commitment of T helper cells to the $T_H1$ lineage via activation of the transcription factor *Signal transducer and activator of transcription 4* (STAT4). STAT4, in turn, activates the expression of several $T_H1$-specific genes, including *IFNG* and, most importantly, *TBX21* which encodes for the transcription factor *T-box transcription factor TBX21* (TBET) considered the master regulator of this lieneage[131]. TBET expression is additionally promoted by IFN-γ itself (via STAT1) creating a coordinated positive feedback loop for further TBET and *IL-12 receptor* (IL-12R) expression[119]. These cells are major producers of IFN-γ and are involved in the immunity against intracellular microorganisms such as viruses. Type 2 helper cells, on the other side, secrete IL-4, IL-5, and IL-13 and are required for humoral immunity to clear intestinal helminths and other extracellular pathogens[118]. Moreover, $T_H2$ cells are able to promote antibody class-switching to IgE which are involved in allergic reactions[132]. IL-4 is required for the differentiation of this subsets via induction of STAT6 and subsequent activation of the lineage-specific transcription factor G*ATA binding protein 3* (GATA-3)[120, 133].

The $T_H1/T_H2$ dichotomic paradigm of CD4 differentiation was expanded with the identification of the $T_H17$ cells in 2005[134]. These cells, whose key effector are IL-17A, IL-17F and IL-22[135], provide protection against extracellular bacteria and fungi, especially at mucosal surfaces[136] and have been associated with autoimmune disorders and excessive inflammation[137]. Commitment to the $T_H17$ fate in human requires *Transforming growth factor* β (TGF-β), which acts together with IL-1β and STAT3-activating cytokines such as IL-6, and IL-21 or IL-23[138]. TGF-β is essential in inducing *retinoid-related orphan receptor* (ROR-γT, the lineage master transcription factor) and *Chemokine receptor 6* (CCR6; a chemokine receptor characteristic of $T_H17$ cells)[114, 120].

Initially characterized as a subset of $T_H2$ cells, $T_H9$ cells where subsequently defined as a distinct population producing IL-9 (and also IL-10) independently by other $T_H2$ cytokines. They have been associated with allergic reactions, asthma, antitumor immunity and parasitic infections[139]. $T_H9$ cells develop though a differentiation program similar to that of $T_H2$ cells in which, however, TGF-β is required for their final maturation and diverts the differentiation from a $T_H2$ phenotype to $T_H9$[114, 140].

Several studies in humans have investigated $CD4^+$ T cells that primarily secrete IL-22 (but not IFN-γ, IL-4 or IL-17) and recognized them as a distinct additional cell population termed $T_H22$[141]. The precise mechanism inducting differentiation of $T_H22$ cells has not been fully clarified but this newly identified subset has been shown to participate in epidermal repair and

remodelling and it is involved in preventing translocation of microbial pathogens through the epithelial surfaces[142, 143].

Finally, T regulatory cells ($T_{reg}$) are indispensable guardians of peripheral self-tolerance and prevention of autoimmunity. Inducible $T_{reg}$, can be generated in response to various endogenous stimuli, such as apoptotic cells and self-antigens, but also in response to pathogens or inflammation[144]. *Forkhead box P3* (FOXP3), is the key transcription factor defining commitment to this lienage[120]. Despite ongoing debate, $T_{reg}$ are thought to suppress inflammatory immune responses to commensal microbes through various mechanism including the secretion of anti-inflammatory cytokines, such as TGF-β and IL-10[145].

## Characteristics and development of T-follicular helper ($T_{FH}$) cells

Long-lived antimicrobial antibodies responses form the basis of protection afforded by most vaccines. Early evidence that T cell *help* to B cells is necessary for the development of long-lived humoral immunity came from a series of seminal studies in the 1960s[146, 147]. These discoveries fuelled intensive research in the biology of the helper T cells in the quest of identifying the cellular signals and interactions promoting humoral immunity. $CD4^+$ T cells were subsequently found to be essential for the establishment and maintenance of *germinal centres* (GC), the specialized and complex microstructures within secondary lymphoid organs, where high affinity B cells are selected to become antibody secreting plasma cells or memory B cells[148]. Following the early characterization of $T_H1$ and $T_H2$ $CD4^+$ helper cells subsets, the precise nature of the T cell population governing GC reactions remained elusive[149]. In the early 2000s, two independent studies revealed that T cells expressing the follicular homing *C-X-C chemokine receptor type 5* (CXCR5) preferentially induced B cells to produce antibodies *in vitro*[150, 151]. Due to their unique helper function and their location within GC, these newly identified cells where subsequently termed follicular helper cells or $T_{FH}$ cells[152] .

Our knowledge about the biology and pathology of $T_{FH}$ cells has increased dramatically since then, especially with regards to their developmental requirements, transcriptional regulation, expression of surface markers and their role during the highly orchestrated germinal centre reaction within the lymphoid follicles[121]. Moreover, increased interest in the field has shed new light on the role of $T_{FH}$ cells in vaccine immune responses, in primary and acquired immunodeficiencies, and in autoimmunity.

Initial gene expression profiling and characterization of secreted cytokines[153] provided unequivocal evidence that $T_{FH}$ cells are an independent subset, distinct form other the major

$CD4^+$ helper cells linages $T_H1$, $T_H2$ and $T_H17$. A set of highly expressed lineage-specific genes was characterized including *Inducible T cell Costimulator* (ICOS), *Achaete-Scute family bHLH transcription factor* 2 (ASCL2), IL-21, P*rogrammed cell death protein 1* (PD-1) and *B cell lymphoma 6* (BCL6)[146, 153]. The latter in particular, was subsequently found to be an indispensable transcription factor for $T_{FH}$ development, whose absence abrogates GC formation. BCL6 was therefore designated as the transcription factor defining the $T_{FH}$ lineage[146, 154]. Conversely, GC $T_{FH}$ cells express neither *TBX21*/TBET, GATA3 or RORγT, the master transcriptional regulators of $T_H1$, $T_H2$ and $T_H17$ cells respectively[146, 155]. However, polyfunctional T helper cell subsets may arise during inflammatory conditions and infections, and $T_{FH}$ cells with various cytokine profiles have been described[156]. $T_{FH}$ biology is still a dynamic field, and it remains to be seen whether specialized subsets of $T_{FH}$ cells exist, or whether these cells rather represent dynamic polarization states of the same lineage.

Currently, human $T_{FH}$ cells are reliably defined by the production of IL-21 and positive surface expression of CXCR5, PD-1, ICOS, *CD40 ligand* (CD40L) and OX40, and the expression of BCL6[146, 149]. The development of $T_{FH}$ cells is a complex multistage process highly dependent on the microanatomical environment within secondary lymphoid follicles. The differentiation of $T_{FH}$ cells is subject to tight spatiotemporal control mechanisms integrating a multitude of molecular signals[152].

Antigen-loaded dendritic cells and monocytes[157], which have migrated to the lymphoid tissues are responsible for the initial priming of naïve $CD4^+$ T cells to undergo $T_{FH}$ differentiation[158, 159] (***Fig. 1***). Persistent antigen presentation[160], combined with co-stimulatory molecules and cytokine signals[114], the paradigmatic three signals, enable professional APCs to activate cognate T cells. T cells expressing TCRs with higher affinity for peptide bound MHC class II have been shown to preferentially undergo $T_{FH}$ differentiation [161]. It is reasonable to assume that higher TCR affinity promotes prolonged APC-T cell contact increasing the exposure of naïve $CD4^+$ to costimulatory signals and APC secreted cytokines thus providing an explanation for this apparent requirement[121].

$T_{FH}$ cells in secondary lymphoid organs exist, as mentioned, in various stages of differentiation characterized by different levels of expression of their characteristic markers[152]. This spectrum reflects the different anatomical localizations of $T_{FH}$ cell differentiation from initial APCs-mediated activation in the T cell zone, to interaction with cognate B cells at the T-B cell border (pre/early $T_{FH}$) to finally supporting proliferation and differentiation of B cells within the GC itself (GC $T_{FH}$)[162, 163].

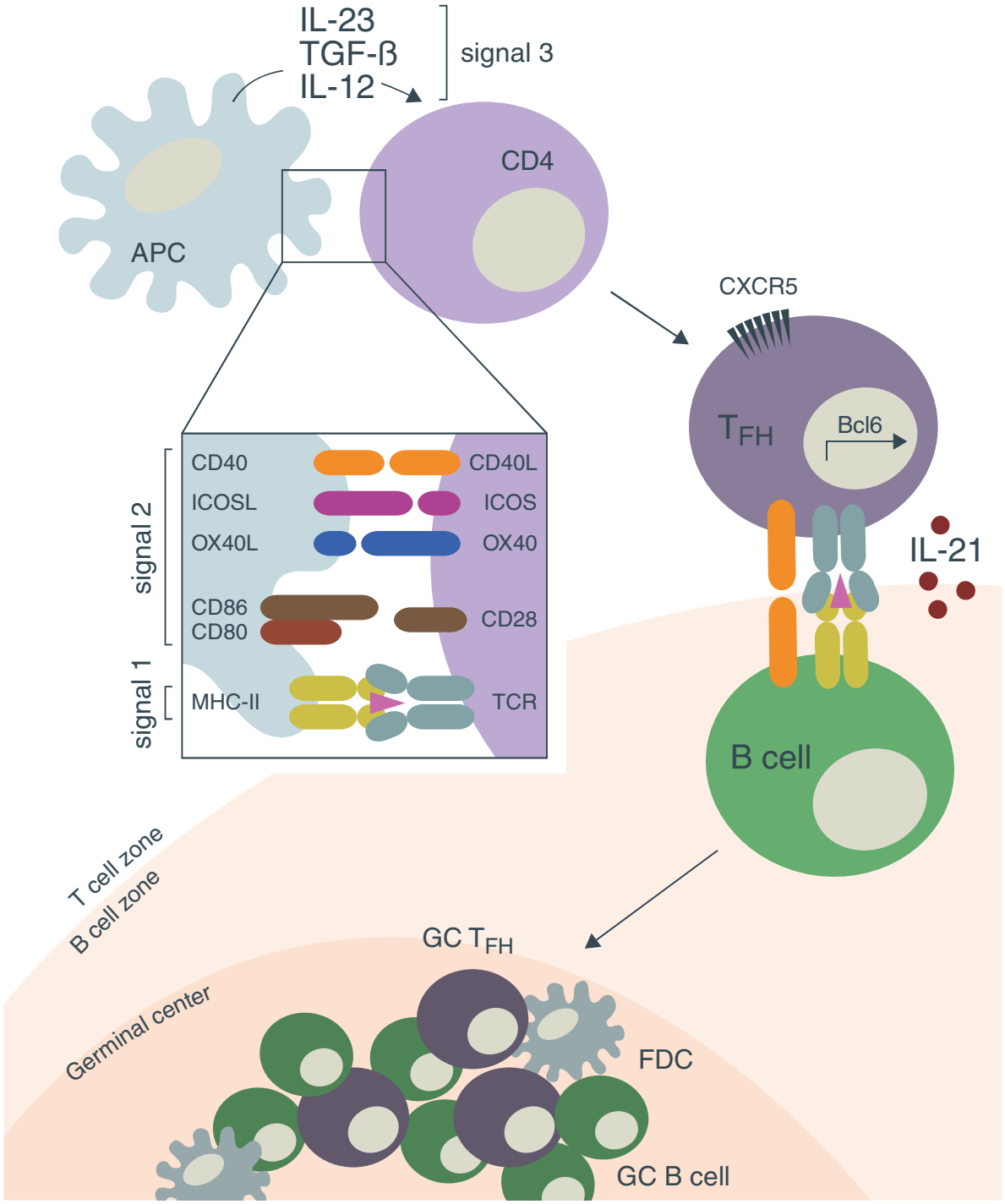


***Figure 1. Schematic representation of the events associated with $T_{FH}$ cells differentiation***. In the T cells zone of the lymph node APCs first interact with naïve CD4 T cells providing the three signals necessary for differentiation into the TFH subset. This include antigen-presentation, costimulatory molecules and secreted cytokines (detailed molecular cell-to-cell interactions are highlighted in the box). Following activation IL-21 producing pre-TFH migrate to the T:B cell border where they engage with cognate B cells concomitantly supporting B cells stimulation and stabilizing their own full development into the follicular helper phonotype. B cells can migrate into follicles to promote the germinal centre reaction. GC TFH cells continue to provide help to the B cells, supporting the generation memory B cells and long-lived plasma cells. Within the GC, B cells proliferate and undergo several rounds of somatic hypermutation. GC B cells whit high affinity BCR can collect antigens from follicular dendritic cells (FDC) and present them to GC TFH to support their survival. At the same time B cell signals are also fundamental for sustaining TFH cells.

Cytokines play a key role during $T_{FH}$ differentiation, and these differ substantially between humans and mice[146]. While IL-6 and IL-21 have a prominent role in mice[146, 155], in humans, the major cytokines driving $T_{FH}$ differentiation are considered to be Il-12, IL-23 and TGF-β (with a less prominent role for IL-6 and IL-1β[164]). These cytokines synergize to promote the early $T_{FH}$ phenotype by inducing the expression of key genes BCL6, ICOS, IL-21 and CXCR5[164-166]. Confirming the central role of IL-12, individuals carrying a functional deficiency of the IL-12 receptor β1 chain (the common receptor for both IL-12 and IL-23) have fewer circulating memory $T_{FH}$ cells, fewer blood memory B cells, altered GC formation in lymph nodes, and anti-tetanus toxoid immunoglobulins from these individuals show a markedly reduced affinity[167, 168].

Activated APCs also provide costimulatory signals in the form of specific receptor/ligand pairs engaging at the interface with T cells, the immunological synapse. Professional APCs express ICOSL[169], OX40L[170], CD80/CD86[171] and CD40[172], which bind to their interaction partners on the T cell surface, ICOS, OX40, CD28 and CD40L respectively[162, 169]. The requirement for co-stimulation during $T_{FH}$ differentiation has been demonstrated by several studies, and reduced $T_{FH}$ frequencies have been observed in carriers of monogenic mutations impairing these molecular interactions [168].

Upon cognate interaction with APCs, a hierarchical network of transcription factors is activated in naïve $CD4^+$ T cells skewing their phenotype towards the $T_{FH}$ pathway. BCL6 has a fundamental role and promotes $T_{FH}$ differentiation as a transcriptional repressor interfering with factors determining alternative T helper fates[146]. BCL6 inhibits the activity of *B-Lymphocyte-Induced Maturation Protein 1* (BLIMP-1), which is induced via the IL-2-STAT5 axis in $CD4^+$ T cells and antagonizes $T_{FH}$ development[173]. Moreover, the targets of its repressive activity also include promoters of genes involved in $T_H1$ (i.e. *TBX21*), $T_H2$ (i.e. *GATA3*) or $T_H17$ (i.e. *RORγT*) differentiation[174]. As a positive regulator of $T_{FH}$ cells development, BCL6 together with ASCL2 is involved in the upregulation of chemokine receptor CXCR5 and concomitant downregulation of *C-C chemokine receptor type 7* (CCR7) and the adhesion molecule *P-selectin glycoprotein ligand-1* (PSGL-1). The coordinated regulation of these molecules allows $T_{FH}$ cells to relocate from the T cell zone (periarteriolar sheath) to the B cell area of the follicles, where they can interact directly with cognate B cells[146]. Other transcription factors also play a role $T_{FH}$ differentiation; these include MAF (involved in ICOS dependent IL-21 expression[154]), *Basic Leucine Zipper ATF-Like Transcription Factor* (BATF, which is required to induce both BCL6 and MAF[175]) and IRF4, which has been recently shown to tip the balance of $T_{FH}$/ $T_H1$ differentiation towards $T_{FH}$ commitment[176]. Moreover, cytokines that induce IL-21 in naïve human $CD4^+$ T cells are known to trigger the JNK/STAT pathway and

different members of this family of transcription factors are phosphorylated and activated as a consequence: this include, most importantly, STAT3 and STAT4 which both act downstream of TGF-β and IL-12[164, 177].

However, the upstream signals, e.g. microbial stimuli that license APC to initiate $T_{FH}$ cell differentiation remain largely unclear.

## Roles of $T_{FH}$ cells in health and diseases

Following initial activation by APCs and upregulation of CXCR5, early $T_{FH}$ committed cells move toward the T:B cell border where a second round of signals provided by B cells is necessary for survival and maintenance of the established $T_{FH}$ phenotype[155]. While B cells are not required to initiate $T_{FH}$ development, at this stage, antigen specific B cells, which are usually rare, have proliferated and become the major antigen presenting population within the follicle forming a 'symbiotic' relationship with their cognate T cells[155]. The B:$T_{FH}$ interaction, providing further and continual antigen stimulation[121, 160], is essential for terminal $T_{FH}$ cell differentiation and survival, and represents the key event triggering GC formation[178].

Activated B cells express other costimulatory signals required for the development of GC $T_{FH}$ cells including CD80, CD86 and ICOSL[146]. At the same time T cells provide critical help to the B cells via CD40L, IL-21, and ICOS. CD40L-CD40 signals prevent B cell apoptosis and promote proliferation. In combination with cytokines, CD40L-CD40 signals also promotes isotype switching and differentiation of plasma cells[179]. A hallmark feature of $T_{FH}$ cells is their ability to produce high levels of IL-21, potent inducer of plasma cell differentiation of CD40L-stimulated B cells[180]. Therefore, following engagement at the T:B border, B cells, which now express BCL6[155], are fully licensed to divide and are able to further differentiate into the extrafollicular pathway, forming short lived plasmablasts, or give rise to a nascent GC. Interaction with antigen-presenting B cells leads to a secondary increase of BCL6 in T cells, which in turn stabilises CXCR5 expression and terminal differentiation into mature GC $T_{FH}$ cells. Aided by B cell-dependent ICOS signalling[181], $T_{FH}$ cells migrate into the GC[146, 181], where they fulfil their critical helper function and promote memory B and plasma cell differentiation.

The most important role of $T_{FH}$ is unquestionably their unique ability to promote GC formation. Within the GC, B cells can undergo multiple rounds of somatic hypermutation and selection, which will ultimately generate cells secreting antibodies with a high affinity for a pathogen or against a vaccine antigen[146, 148, 155]. In humans, the architecture of the GC is composed by two areas defined as *light zone* and *dark zone* (LZ and DZ, respectively). B cells

undergo somatic hypermutation in the variable region of their immunoglobulin genes allowing antibody diversification in a process that predominantly occurs in the dark zone, the region closer to the T zone[121]. Following several rounds of proliferation, GC B cells migrate to the light zone. Here, B cells, which have acquired BCR with increased affinity are more likely to pick up unprocessed antigens held by *follicular dendritic cells* (FDCs) and present them in the context of MHC class II to $T_{FH}$ cells and compete for their help[155]. In turn, GC $T_{FH}$ cells provide signals to B cells to foster their survival and proliferation[146].

T:B cell interaction, as highlighted, is not unidirectional; in fact, production of IL-21 is stimulated by ICOSL expressed on B cells and IL-4 secretion is selectively promoted in a *Signaling lymphocytic activation molecule* (SLAM) depended manner[121]. B cells that fail to receive survival signals in the LZ are eliminated *in situ* by apoptosis, and are cleared by tingible body macrophages[146]. B cells can undergo three distinct differentiation fates after selection by cognate T cells: (i) differentiation into (long lived) plasma cells; (ii) differentiation into long-lived memory B cells; (iii) migration the DZ and undergo further affinity maturation. The signals promoting plasma cell and memory B cell differentiation are comparably better understood than those promoting additional selection[146]. In mice, IL-21, IL-4, IL-6, and IL-10 coupled with decreased CD40L signalling have been implicated in the formation of plasma cells[146, 155]. It is noteworthy that $T_{FH}$ cells are also involved in other important immune processes beyond GC formation, and it was shown that ICOS dependent $BCL6^+$ cells are also involved in stimulating primed B cells along the extrafollicular pathway[182].

Being a central resource in the generation of persisting high affinity B cell responses, it is not surprising that $T_{FH}$ cells have been associated with protective immunity against pathogens and control of commensal bacteria, but also in the pathogenesis of a range autoimmune diseases and, surprisingly, cancer immunosurvellance[183, 184]. Dysfunctional $T_{FH}$ responses are observed in HIV infected individuals where, paradoxically, an abnormal expansion of $T_{FH}$ cells leads to increased antibody responses (i.e. hypergammaglobulinemia), however undermining the ability to generate broadly neutralizing antibodies to counter fight the extreme mutability of the virus[184, 185]. Numerous monogenic primary immunodeficiencies correlated with dysregulated $T_{FH}$ development or maintenance are traceable to genetic defects in several of the aforementioned key factors necessary for the generation of functional $T_{FH}$ cells, including mutations in *SLAM-associated protein* (SAP; associated with X-linked lymphoproliferative disease), CD40L (which causes hyper-IgM syndrome) or ICOS (involved in adult-onset common variable immunodeficiency)[146, 184].

Many studies have found that a tight regulation of $T_{FH}$ responses prevents the generation of autoantibody and, as a consequence, $T_{FH}$ overactivation is thought to play a direct pathogenic role in several autoimmune diseases such as *systemic lupus erythematosus* (SLE), juvenile dermatomyositis, Sjögren's syndrome and autoimmune thyroid disease[121].

Most importantly, $T_{FH}$ cells have a fundamental role in long-term protective immunity elicited by human vaccines[186]. However, the targeted activation of $T_{FH}$ signals has proven a difficult task. Vaccine adjuvants are the most obvious means of boosting $T_{FH}$ responses, or to interfere with $T_{FH}$ inhibitory pathways. Targeted harnessing of $T_{FH}$ cells would have a significant impact on the design and development of next-generation vaccines with the aim of inducing sustained protection against infection[187].

## 1.7 Aims of this study

The aim of the current study was to investigate the largely unknown innate immune mechanisms, which might explain the aforementioned remarkable ability of live attenuated vaccines to promote lifelong protective immunity. The recognition of bacterial viability had been previously investigated in depth in the murine model[99], but the impact of *vita*-PAMPs, their nature, and their sensing receptor in human are yet to be characterized. Understanding these mechanisms would provide valuable insights into fundamental events of innate immune recognition and its consequences for the adaptive immune responses, which are at the heart of efficacious vaccines. The early events of vaccine responses and particularly their effect on $T_{FH}$ cell differentiation induced by activated APCs, remain incompletely understood, especially in humans. We aimed, therefore, to investigate the mechanisms of 'viability recognition' in human APCs and to dissect their impact on downstream T helper cell and vaccine responses.

The present study reveals that the sensing of bacterial viability, via detection of bacterial RNA through TLR8, is a highly conserved innate immune checkpoint fundamental in orchestrating the adaptive arm of the immune system towards the generation of $T_{FH}$ cells and humoral immunity.

These results expand our current knowledge about innate control of adaptive immune responses, and they provide valuable new targets for the rational design of much needed new vaccines adjuvants.

# 2 MATERIALS & METHODS

## 2.1 Reagents, materials and instruments

### Reagents

| *Reagent* | *Manufacturer* |
|---|---|
| 10% neutral-buffered formalin (4% formaldehyde) | Pioneer Research Chemicals Ltd |
| Ammonium chloride ($NH_4Cl$) | Sigma-Aldrich |
| Ampuwa water | Fresenius Kabi |
| Bacto Agar | BD Biosciences |
| Bacto Tryptone | BD Biosciences |
| Bacto Yeast extract | BD Biosciences |
| Bafilomycin | Sigma-Aldrich |
| Bovine Serum Albumin (BSA) | Miltenyi Biotec |
| Brefeldin-A | eBioscience |
| Chloroform | Sigma-Aldrich |
| Citric Acid | Sigma-Aldrich |
| CL075 | Invivogen |
| Concanavalin A | Sigma-Aldrich |
| CpG-ODN 2395 | Invivogen |
| Cytochalasin D | Sigma-Aldrich |
| Cytofix/Cytoperm Fixation and permeabilization Solution | BD Biosciences |
| D-Glucose | Sigma-Aldrich |
| dH2O | Merck Millipore |
| Diaminobenzidine | Sigma-Aldrich |
| Dimethyl sulfoxide (DMSO) | Sigma-Aldrich |
| Ethanol | Merck |
| Ethylenediaminetetracetic acid (EDTA) | Sigma-Aldrich |
| Fetal Calf Serum (FCS) | Sigma-Aldrich |
| GlutaMax | Life Technologies |
| Glycerol | Sigma-Aldrich |
| Glycoblue | Thermo Fisher |

| | |
|---|---|
| Hank's Balanced Salt Solution (*HBSS)* | Thermo Fisher |
| Hematoxylin | Sigma-Aldrich |
| Heparin | Sigma |
| HEPES buffer | Sigma-Aldrich |
| Histopaque-1077 | Sigma-Aldrich |
| Human Serum | Millipore |
| IMDM | PAN-Biotech GmbH |
| Ionomycin | Sigma-Aldrich |
| Isopropanol | Sigma-Aldrich |
| Leibovitz's L-15 Medium | Lonza |
| L-Glutamine | Lonza |
| LPS-EK ultrapure | Invivogen |
| Middlebrook 7H9 broth base powder | Sigma-Aldrich |
| Middlebrook OADC growth supplement | Sigma-Aldrich |
| Monensin | BioLegend |
| Monophosphoryl Lipid A (MPLA)- VacciGrade | Invivogen |
| MyD88 siRNA SilencerSelect | Life Technologies |
| Non-Essential Amino Acids | Sigma-Aldrich |
| Nuclease-Free Water | Thermo Fisher |
| Pam3CSK4 | Invivogen |
| Pancoll (1.077g/ml) | PAN-Biotech GmbH |
| Paraformaldehyde | Sigma-Aldrich |
| Penicillin/Streptomycin | Sigma-Aldrich |
| Percoll | GE Healthcare |
| Perm/Wash buffer | BD Biosciences |
| Phorbol 12-myristate 13-acetate (PMA) | Sigma-Aldrich |
| Phosphate Buffer Saline (PBS) | Gibco |
| Poly(I:C) LMW | Invivogen |
| Poly-L-arginine (pLa) | Sigma-Aldrich |
| Potassium bicarbonate ($KHCO_3$) | Sigma-Aldrich |
| R848 | Invivogen |
| Red Blood Cells Lysis Buffer | Sigma-Aldrich |
| RNase-free DNase I | Sigma-Aldrich |
| RPMI1640 | Gibco |
| Silencer Select Negative Control No. 1 siRNA | Life Technologies |
| Sodium Bicarbonate ($NaHCO_3$) | Sigma-Aldrich |
| Sodium Chloride (NaCl) | Sigma-Aldrich |
| Staphylococcal-Enterotoxin-B (SEB) | Sigma-Aldrich |
| Streptavidin | BioLegend |
| Streptavidin-HRP | Thermo Fisher |
| Streptocillin vet. | Boehringer Ingelheim Vetmedica |
| Sulfuric Acid ($H_2SO_4$) | Sigma-Aldrich |
| Thymidine | Sigma-Aldrich |
| TLR8 siRNA SilencerSelect | Life Technologies |
| TMB Substrate Solution | TMB Substrate Solution |
| Trimethoprim | Sigma-Aldrich |
| TRIzol Reagent | Thermo Fisher |
| Trypan Blue | Sigma-Aldrich |
| Tween-20 | Sigma-Aldrich |
| Tween-80 | Sigma-Aldrich |
| Viromer Blue transfection reagent | Lipocalyx |

| | |
|---|---|
| Xylene | Sigma-Aldrich |

## Materials

Standard laboratory consumables including pipette tips, conical centrifuge tubes, microcentrifuge tubes, serological pipettes etc. are not reported here. Generally, cell culture flasks, plates and dishes, bacterial petri dishes and centrifuges tubes were purchased from Corning.

Special materials used are listed here:

| *Material* | *Manufacturer* |
|---|---|
| 2ml, Nunc CryoTubes | Thermo Scientific |
| Disposable filters (pore size 0.22µm, Ø 33mm) | Millipore |
| Falcon 96-well Clear Round Bottom TC-Treated Cell Culture Microplate | Corning |
| Falcon Cell Strainers (mesh size 70µm) | |
| Falcon Round-Bottom Polystyrene Tubes | Corning |
| Nunc Clear Flat-Bottom Immuno Nonsterile 96-well Plate | Thermo Scientific |
| Tuberculin Syringe | BD Biosciences |

## Kits

| *Kit* | *Manufacturer* |
|---|---|
| Affymetrix GeneChip Human Gene 1.1 ST | Thermo Fisher |
| Ambion WT Expression Kit | Thermo Fisher |
| Antibody pair for human IL-12p40 ELISA: | |
| Capture Antibody, (C8.3) | eBioscience |
| Detection Antibody (C8.6) | eBioscience |
| Recombinant IL-12/23p40 | BioLegend |
| CD14 MicroBeads, human | Miltenyi Biotec |
| ColorRapid | Lucerna Chem AG |
| EasySep monocyte isolation with CD16 depletion | StemCell Technologies |
| Foxp3 / Transcription Factor Staining Buffer Set | eBioscience |
| GeneJET RNA Purification Kit | Thermo Fisher |
| GenElute Mammalian Total RNA Miniprep Kit | Sigma-Aldrich |
| Human GM-CSF beta ELISA Ready-SET-Go! | eBioscience |
| Human IL-1 beta ELISA Ready-SET-Go! | eBioscience |
| Human IL-10 beta ELISA Ready-SET-Go! | eBioscience |
| Human IL-21 ELISA MAX Deluxe | eBioscience |
| Human IL-23 Platinum ELISA | eBioscience |

| | |
|---|---|
| Human IL-6 beta ELISA Ready-SET-Go! | eBioscience |
| Human TNF alpha ELISA Ready-SET-Go! | eBioscience |
| IL-12 p70 Human ELISA Kit, High Sensitivity | eBioscience |
| MagniSort Human CD4 Naïve T cell Enrichment Kit | eBioscience |
| MagniSort Human CD4 T cell Enrichment Kit | eBioscience |
| Porcine IL-12/IL-23 p40 Quantikine ELISA Kit | R&D Systems |
| Porcine IL-6 Quantikine ELISA Kit | R&D Systems |
| ProcartaPlex Pig Simplex Kit IL-12/IL-23 p40 | eBioscience |
| ProcartaPlex Pig Simplex Kit IL-6 | eBioscience |
| ProcartaPlex Pig Simplex Kit TNFα | eBioscience |
| ProcartaPlex Porcine Basic kit | eBioscience |
| qScript cDNA Synthesis Kit | Quanta Bioscience |
| RevertAid First Strand cDNA synthesis Kit | Thermo Fisher |
| RNeasy Micro Kit | Qiagen |
| SYBR Green Jumpstart Taq ready mix kit | Sigma-Aldrich |
| SYBR Select Master mix | Thermo Fisher |
| TRIzol Max Bacterial RNA Isolation Kit | Thermo Fisher |

## Buffers and cell culture media

| *Buffer* | *Composition* |
|---|---|
| Cell culture media | RPMI1640<br>10% FCS<br>1% Non-Essential Amino Acids<br>1% GlutaMax<br>1% HEPES |
| Cell freezing medium | FCS<br>10% DMSO |
| EasySep and FACS Buffer | PBS<br>2% FCS<br>1mM EDTA |
| ELISA wash buffer | PBS<br>0.05% Tween-20 |
| Erythrocyte lysis solution (animal exp.), pH 7.5 | $dH_20$<br>0.01M $KHCO_3$<br>0.155M $NH_4Cl$<br>0.1mM EDTA |
| LB Agar | LB medium, pH 7.5<br>10% Bacto Agar |
| Luria-Bertani (LB) Medium, pH 7.5 | For 1L of $dH_2O$<br>5g Bacto Yeast Extract<br>10g Bacto Tryptone<br>10g NaCl |

| | |
|---|---|
| MACS Buffer | PBS<br>0.5% BSA<br>2mM EDTA |
| MagniSort Buffer | PBS<br>3%FCS<br>10mM EDTA |
| Middlebrook 7H9-T | 450ml $dH_2O$<br>2.35g Middlebrook 7H9 Broth Base<br>0.5g Tween-80<br>1 vial Middlebrook Growth Supplement |
| Porcine splenocytes culture media | IMDM<br>10% FCS |
| RPMI-EDTA wash buffer | RPMI1640<br>5% FCS<br>2mM EDTA |
| T cell culture media | RPMI1640<br>10% human serum<br>1% Non-Essential Amino Acids<br>1% GlutaMax<br>1% HEPES |

## Antibodies

| *Antibodies (clone)* | *Manufacturer* |
|---|---|
| anti-human BCL6 (K112-91) | BD Biosciences |
| anti-human CD115 (12-3A3-1B10) | eBioscience |
| anti-human CD14 (61D3) | eBioscience |
| anti-human CD16 (eBioCB16) | eBioscience |
| anti-human CD197 (CCR7) (G043H7) | BioLegend |
| anti-human CD28 (CD28.2) | BioLegend |
| anti-human CD3 (UCHT1) | BioLegend |
| anti-human CD4 (OKT4) | BioLegend |
| anti-human CD40 (5C3) | BioLegend |
| anti-human CD45RA (HI100) | BioLegend |
| anti-human CD80 (2D10) | BioLegend |
| anti-human CD86 (IT2.2) | BioLegend |
| anti-human HLA-DR (L243) | BioLegend |
| anti-human ICOSL (2D3) | BioLegend |
| anti-human IL-21 (eBio3A3-N2) | eBioscience |
| anti-human OX40L (11C3.1) | BioLegend |
| anti-human Pax-5 (24/Pax-5) | BD Biosciences |
| anti-swine BCL6 (orb234772) | Biorbyt |
| anti-swine CD14 (Tük4) | Miltenyi Biotec |
| anti-swine CD4 (74-12-RUO) | BD Biosciences |
| anti-swine IgG (H+L) | Seracare |

| | |
|---|---|
| anti-swine IL-21 (orb9043) | Biorbyt |
| anti-swine Monocyte/granulocyte (CD172; 74-22-15A) | BD Biosciences |
| Goat anti-rabbit Ig (BA 1000) | Vector |
| Goat anti-mouse Ig (BA9200) | Vector |

### Instruments

Standard laboratory equipment including, but not limited to, automatic pipettors, cell and bacterial incubators, fumes and laminar flow hoods, centrifuges, workbenches, water baths, ice machines, refrigerators etc. are not listed here. Those instruments, however, are in accordance with national laboratory standards and are regularly maintained. Only special instruments used are reported here.

| *Instruments* | *Manufacturer* |
|---|---|
| ABI 7300 Real-Time PCR System | Applied Biosystems |
| Accuri C6 PLUS flow cytometer | BD Biosciences |
| Affymetrix GeneTitan Instrument | Thermo Fisher |
| Agilent 2100 Bioanalyzer | Agilent |
| Aperio CS2 digital pathology scanner | Leica |
| BioPhotometer UV/Vis Spectrophotometer | Eppendorf |
| C1000 Thermal cycler | BioRad |
| *CASY* cell counter | Innovatis |
| FACSAria II SORP cell sorter | BD Biosciences |
| FACSAria III cell sorter | BD Biosciences |
| FACSCanto II cytometer | BD Biosciences |
| FilterMaxF5 Multi-Mode Microplate Reader | Molecular Devices |
| Luminex MAGPIX instrument | Millipore |
| NanoDrop 2000 Spectrophotometers | Thermo Fisher |
| PTC-200 Peltier Thermal Cycler | MJ Research Inc. |
| Shandon Cytospin III | Shandon Scientific ltd. |

## 2.2 Methods

### Cell isolation and culture

Human monocytes ($CD14^+CD16^-$) and $CD4^+$ T cells used in this study were either freshly isolated from leucocytes concentrates (buffy coats) purchased from the German Red Cross Blood Transfusion Service, Berlin, Germany or from peripheral venous blood of healthy volunteers.

In either case, permission for experiments with human primary cells was granted by the local ethic committee (Charité – Universitätmedizin Berlin). Briefly, peripheral blood mononuclear cells (PBMC) were isolated by density gradient centrifugation of diluted (1:1 in appropriate buffer) buffy coats, or freshly collected blood, over Histopaque-1077 or Pancoll. The layer containing lymphocytes and mononuclear cells was then harvested and washed three times in the appropriate RPMI-EDTA wash buffer to eliminate contaminating platelets and residual density gradient components. If necessary, an erythrocytes lysis step was performed after the first wash using Red Blood Cell Lysis Buffer. $CD14^+CD16^-$ monocytes (termed classical monocytes) were purified by negative selection via immunomagnetic separation using EasySep Monocyte Isolation kit with CD16 and platelets depletion according to the manufacturer's instructions. Isolated monocytes were cultured at a density of $1 \times 10^6$ cells/ml (for single culture experiments) or at $4 \times 10^5$ cells/ml (for co-culture experiments) in antibiotic-free RPMI1640 supplemented with 10% FCS, 1% glutamine, 1% HEPES buffer, 1% Non-Essential Amino Acids. Flow cytometric analysis were carried out on an Accuri C6 PLUS flow cytometer to assess the purity of the cell preparation using fluorescently labelled antibodies against human CD14 and CD16. Briefly, cells were harvested, washed in FACS buffer and stained in the same buffer for 30min at 4°C with an antibody mixture containing CD14 and CD16 antibodies. Cells where then washed twice in the same buffer and resuspended in FACS buffer until analysis.

Naive $CD4^+$ T cells were isolated by immunomagnetic separation using negative selection MagniSort Human CD4 Naive T cell Enrichment Kit, following manufacturer's instructions. Staining with CD45RA and CCR7 was used to determine the degree of purity of the isolated naïve $CD4^+$ T cells by flow cytometry. Total $CD4^+$ T cells (which were used for experiments in Figs. 27 and 28) were isolated by magnetic separation using an alternative negative selection kit, MagniSort Human CD4 T cell Enrichment Kit according to the manufacturer's guidelines. Their purity was routinely checked by flow cytometry after staining with an anti-CD3 and anti-CD4 antibody mixture. T cells were cultured in RPMI1640 supplemented with 10% human serum (collected from the respective T cell donor), 1% glutamine, 1% HEPES buffer, 1% Non-Essential Amino Acids. All cells were grown at 37°C, 5% $CO_2$ in a humidified incubator.

Exclusively cell preparations with purities greater of 90% (monocytes), 90% (Naïve T cells) or 95% (total T cells) were used for subsequent experiments.

Cell numbers were assessed by cell courting using Neubauer counting chamber and trypan blue exclusion to visualize cell viability. Alternatively, cells were counted using an Accuri C6 PLUS instrument gating on viable and single cells by means of forward scatter (FSC) and side scatter (SSC).

## Bacteria

*Escherichia coli* K12, strain DH5α, thymidine auxotrophs (*thyA*$^-$) were selected as reported previously[1]. Briefly commercially available *Escherichia coli* K12, strain DH5α were plated on Luria-Bertani (LB)-agar plates supplemented with 50μg/ml trimethoprim and 500μg/ml thymidine. After 7 days of incubation at room temperature, single colonies of naturally occurring thymidine auxotrophic (*thyA*$^-$) *E. coli* were carefully isolated and their auxotrophy confirmed by inoculation in liquid LB medium, containing the aforementioned concentration of both trimethoprim and thymidine, and culturing overnight at 37°C. *ThyA*$^-$ *E. coli* (through this work simply referred to as EC) grew only in the presence of thymidine and were resistant to trimethoprim. *Bacillus subtilis* (referred as BS) strain 168 (kindly provided by Dr. Catherine Chapout at the Charité – Universitätmedizin Berlin) were grown in LB medium at 37°C. *Salmonella enterica* serovar Thyphymirium (referred as ST), histidine and thymidine auxotroph, as lyophilized preparation, was a kind gift of Dr. Sven Springer, IDT Biologika Gmbh and commercially available under the trade name Salmoporc®-STM. Bacteria were resuspended in the provided saline solution and pleated overnight on LB agar plates at 37°C. Single colonies were isolated and propagated in LB medium. *Mycobacterium bovis* strain BCG (referred as simply BCG) was grown in Middlebrook 7H9 medium supplemented with 0.05% Tween 80. Aliquots of the all the bacterial strains utilized were prepared in 30% glycerol and stored at -80°C until use.

## Infection experiments

For phagocytosis and infection experiments, EC (or equally BS, ST or BCG) were grown to mid-log phase, pelleted and washed twice in phosphate buffered saline (PBS) to remove residual growth media components and secreted factors. Bacteria were resuspended in saline before addition to the cells at the reported multiplicity of infection (MOI). In the case of BCG, log-phase bacteria were washed twice in PBS and the formation of a single cell suspension in complete cell culture media was ensured through repeated (ten times) tuberculin type needle passages.

For heat killing, EC were grown to mid-log phase, washed and re-suspended in PBS at an optical density at 600nm (OD600) of 0.6, and subsequently incubated at 60°C for 90min. Heat-killed *thyA*$^-$ *E. coli* (HKEC) were used immediately after killing or stored at -80°C in 30% glycerol for up three months. Efficiency of killing was assessed by overnight plating on

thymidine/trimethoprim-supplemented LB-agar plates. For heat-killed *B. subtilis* (HKBS), the bacteria were grown to mid-log phase, washed and re-suspended in PBS at an $OD_{600}$ of 0.6, and subsequently incubated at 95°C for 30min. Efficient killing was evaluated by overnight plating on LB-agar plates. Heat-killed BCG (HKBCG) were grown to log phase and inactivated at 60°C for 90min. Heat-killed BCG were resuspended in cell growth media and used immediately after killing. Efficient killing was confirmed by 96h inoculation in competent media. For heat killed *S. enterica* serovar Typhimurium, log phase bacteria were grown at an optical density at $OD_{600}$ of 0.8, and subsequently inactivated with the same procedure used for *E. coli*. Efficiency of killing was confirmed by overnight plating on LB-agar plates.

Ethanol killing was performed by resuspending log phase *thyA*$^-$ *E. coli* in 70% ethanol for 10 min. For ultraviolet killing log phase bacteria were resuspended at an $OD_{600}$ of 0.6 and irradiated twice with 1000mJ/cm in an uncovered bacterial petri dish. Paraformaldehyde (PFA) fixation was carried out by resuspending log-phase *E. coli* in 2ml of 4% PFA for 15 min. Bacteria inactivated with different methods were then extensively washed (at least four times) before re-suspension in PBS prior to cell infection. Efficiency of killing was confirmed by overnight plating on LB-agar plates.

Infection of human monocyte or porcine monocytes and dendritic cells was performed at a MOI of 10 if not otherwise stated. Immediately following the addiction of bacteria, plated cells were briefly centrifuged for 1min at 2500rpm to ensure simultaneous contact between bacteria and cells. After 1.5 hours, penicillin/streptomycin (1%) was added to inhibit any further replication of bacteria. Supernatants were collected 18h post infection for human samples and 24h post infection for pig samples, and analysed by ELISA for cytokine content.

## Cells stimulation experiments

Purified ligands of human TLRs were used to stimulate isolated human or porcine APCs at the indicated concentration: CL075 (3M002; 1μg/ml), LPS-EK Ultrapure (2μg/ml), Pam3CSK4 (200ng/ml), Poly(I:C) LMW (2μg/ml), ODN 2395 (5μM). Pre-treatment with bafilomycin A (Baf) and cytochalasin-d (CytoD) was performed at a concentration of 1μM for 30min before infection.

Bacterial RNA was isolated from mid-log phase cultures of DH5alpha *E. coli* as described below. Transfection of bacterial RNA into human monocytes was performed using polycationic polypeptide poly-L-arginine (pLa) as previously described[2]. Briefly, 280ng of pLa (2,8ul at 100μg/ml) were mixed with 239ng of purified bacterial RNA (2.39ul at 100μg/ml) and

incubated at room temperature for 20min before stimulation of cells. A total volume of 5.19µl of the complexed mixture was added to 100µl of cell suspension at a density of 1 x $10^6$ cells/ml in a 96 well plate.
Supernatants were collected 18h post infection and analyzed by ELISA for cytokine content.

**Enzyme-linked immunosorbent assay (ELISA)**

TNF, IL-1β, IL-6, IL-10, IL-12p40, IL-23 and GM-CSF concentrations in cell culture supernatants were measured by enzyme-linked immunosorbent assay (ELISA) according to standard manufacturer's recommendations. Concentrations of IL-12p70 were measured using a human IL-12p70 High Sensitivity ELISA kit. The samples were analysed for absorbance at 450 nm using FilterMax F5 Multi-Mode Microplate Reader. Porcine IL-12p40 and IL-6 concentrations in culture supernatants of porcine monocytes and dendritic cells were measured by ProcartaPlex Pig Kit based on a proprietary technology of labelled magnetic microsphere. Results were collected using a Luminex MAGPIX instrument at the Department of Veterinary Medicine of the Freie Universität Berlin. For the porcine siRNA experiments (Fig. 31), cytokine IL-6 and IL-12p40 in the culture supernatants following siRNA treatment and infection (as indicated in the respective figures) were analysed by ELISA using Porcine Quantikine ELISA Kits.

**RNA Isolation**

$CD14^+CD16^-$ human monocytes were sorted by flow cytometry and infected with EC at MOI of 10 or stimulated with HKEC at the same ratio of bacteria to cells. After 6 hours, cells were harvested, washed once in PBS, and lysed in Trizol reagent. Lysed cells were either stored at -80°C or used immediately for downstream nucleic acid isolation. Total RNA was prepared according to the manufacturer's suggested protocol. Briefly, chloroform was added the Trizol-cell lysate at a volume ratio of 1 to 5 and, after centrifugation, the colourless aqueous upper phase containing the RNA was collected. To aid co-precipitation and visualization of precipitated RNA, 5µg of RNase-free glycogen (GlycoBlue) was added to the solution. After addition of ½ volume isopropanol, samples were left 15min at room temperature for nucleic acid precipitation. Precipitated RNA was then washed twice in ice cold 75% ethanol and finally resuspended in a minimum 50µl of Nuclease-free water preheated at 57°C.

Total bacterial RNA was isolated with an analogous procedure using the TRIzol Max Bacterial RNA Isolation kit following manufacturer's instructions. Exclusively mid-log phase *E. coli* were used for RNA isolation. Prior to conventional Trizol RNA isolation bacterial cells were incubated at 95°C for 5min with Max Bacterial Enhancement Reagent, a solution of detergents and chelating agents which aids nucleic acid isolation by promoting protein denaturation and inactivation of bacterial RNases. RNA was resuspended in Nuclease-free water preheated at 57°C and stored at -80°C until use.

Quantitation of isolated RNA was performed measuring absorbance using a Nanodrop 2000 spectrophotometer. Simultaneously, quality of nucleic acid was evaluated by assessment of the ratio of absorbance at 260nm and 280nm ($OD_{260/280}$), and 260nm and 230nm ($OD_{260/230}$) respectively.

## Quantitative RT-polymerase chain reaction (RT-qPCR)

Potential contaminant DNA was removed by treatment of 1μg of RNA with DNase I, RNase-free in a final reaction volume of 10μl. Isolated and DNase treated RNA was transcribed into complement DNA (cDNA) using the RevertAid First Strand cDNA Synthesis Kit after DNase inactivation for 10min at 65°C. One microgram of RNA was retrotranscribed in a final reaction volume of 20μl using Random Hexamer as primers as per manufacturer's instructions.

The synthesized cDNA samples were diluted 1:10 in nuclease free water, processed immediately or stored at -20°C until analysis. RT-qPCR was performed on an ABI 7300 Real-Time PCR System using SYBR Select Master Mix, purified primers (TIB MOLBIOL, 300μM), 2μl of diluted cDNA in a total volume of 20μl. Human β-actin was used as reference endogenous gene.

Primers used in this study are listed below:

| ***Gene name*** | ***Accession n.*** | ***Forward & reverse sequences (5'-3')*** |
|---|---|---|
| *TLR1* | NM_003263.3 | TTTTGTGGCCAGGGTCTTCA<br>TGTAGGGGTGCCCAATATGC |
| *TLR2* | NM_001318787.1 | CTCGGAGTTCTCCCAGTGTT<br>AGCCCCACAGGTACCTTCA |
| *TLR3* | NM_003265.2 | AGTGCCGTCTATTTGCCACA<br>TGATTCTGTTGGATGACTGCT |
| *TLR4* | NM_138554.4 | GCGTGGAGGTGGTTCCTAATA |

| | | |
|---|---|---|
| | | TTGAGAAGGGGAGGTTGTCG |
| *TLR5* | NM_003268.5 | TGATGTTCATGTTCCTGACACT<br>AGCATCCCTGGTTTGGTGAC |
| *TLR6* | NM_006068.4 | GGATAGCCACTGCAACATCA<br>TCCGTCGGAGAACTGGATTC |
| *TLR7* | NM_016562.3 | TGCCATCAAGAAAGTTGATGCT<br>GTGTCCACATTGGAAACACCAT |
| *TLR8* | NM_016610.3 | AGTTTCTCTTCTCGGCCACC<br>ACATGTTTTCCATGTTTCTGTTGT |
| *TLR9* | NM_017442.3 | CCCCCAGCATGGGTTTCTG<br>TGGAGCTCACAGGGTAGGAA |
| *TLR10* | AF296673.1 | AACGGAGACATGGCACAGTAG<br>AGATGAGCTCAAAACCCTGTAT |
| *ACTB* | NM_001101.4 | GGATGCAGAAGGAGATCACT<br>CGATCCACACGGAGTACTTG |

The cycling conditions were as follows: 2 min at 50°C, then for 10 min at 95°C followed by 40 amplification cycles (15 sec at 95°C, then 1 min at 60°C).

Melting curve analyses were performed to ensure the specificity of the primers and a two-fold dilution curve cDNA was made for efficiency calculations. Reactions were performed in duplicates and expression raw data were calculated using automatic baseline and automatic Ct and data analysis was done using the ΔΔCt method. Calculated $C^t$-values for each target gene were normalized against the reference *ATCB*.

## Gene Array

Total RNA was prepared from four independent experiments (i.e. four separate donors) according to the aforementioned Trizol manufacturer's protocol. For enhanced nucleic acid quality, samples were further purified on columns RNeasy Micro Kit following manufacturer's specifications. RNA integrity was checked on an Agilent 2100 Bioanalyzer with 6000 Nano Chips. RNA was considered suitable for further analyses only if samples showed intact bands of 18S and 28S ribosomal RNA subunits, displayed no significant chromosomal peaks or signs of RNA degradation products, and had an RNA integrity number (RIN) above 8.0. One-hundred nanograms of RNA were used for whole-transcript cDNA synthesis with the Ambion WT

expression kit according to manufacturer's protocol. An Affymetrix GeneChip Human Gene 1.1 ST 24-array plate was used in this study. Hybridization, wash and scanning steps were carried out according to standard Affymetrix protocols on a GeneTitan instrument.

Quality control, statistical analyses and normalization of signals were performed using the pipeline software MADMAX (a collection of integrated Bioconductor packages)[3]. The different probe sets were defined according to Dai *et al.* using current genome annotations[4]. Estimates of normalized gene expression were calculated from the chip raw intensity values using the robust multiarray analysis pre-processing algorithm within the library "AffyPLM" using default settings[5]. A filtering approach was the applied as follows: only genes that were targeted by at least 7 probes, reached $\log_2$ expression level of at least >4.32 on at least three microarrays and had a $\log_2$ interquartile range value >0.25 across all samples were considered for further downstream analysis. To identify differentially regulated genes, intensity-based moderated t-statistics were used for pairwise comparisons[6]. To correct for multiple testing a false discovery rate method was used to calculate q-values[7]. A stringent q-value < 0.01 was considered significant.

Visualization of heat-map from gene array data was performed using MeV (Multiple Experiment Viewer, Ver. 4.9.0). Scatter plot was generated form raw data using the open-source integrated development environment for R, Rstudio.

The gene array data are currently available to the public in the public Gene Expression Omnibus database (GEO) with accession number GSE68255.

## RNA interference

Silencer Select siRNA duplexes targeting TLR8 (sequence ID: s27920, s27921 and s27922), MyD88 (sequence ID: s9136, s9137 and s9138) and negative controls were obtained from Life Technologies. Monocytes cultured in 96-well plates were transfected with 25nM of each siRNA using Viromer Blue transfection reagent following manufacturer's recommendations for sensitive cells and reverse transfection. Briefly for each well of a 96 well plate, 10µl of siRNA duplexes, diluted at 250nM in provided Buffer F, were combined with an equal amount of Viromer Blue, diluted in the same buffer at 500µM. The mixture was then incubated at room temperature for 15min to allow complexation between siRNA and transfection reagent. Eighty microliters of cells, resuspended to a density of $6.25 \times 10^5$/ml, were then added to each well to final amount of $5 \times 10^4$ cells/well in a final volume of 100µl. Cells were incubated at 37°C.

Seventy-five microliters of cell culture media were replaced with fresh medium at 24h and immediately prior to infection. Forty-eight hours post transfection cells were infected or treated as described.

A similar protocol was used for siRNA mediated silencing in porcine $CD14^+$ monocytes isolated as described below. Four custom-designed Silencer Select siRNA duplexes targeting porcine TLR8 were designed with the following sequences:

| | | |
|---|---|---|
| *TLR8-1* | sense | GCAAAUUGAUUUUACCAUUTT |
| | antisense | AAUGGUAAAAUCAAUUUGCTT |
| *TLR8-2* | sense | GAUUUAAGCUUGAACAGUATT |
| | antisense | UACUGUUCAAGCUUAAAUCTA |
| *TLR8-3* | sense | GCAUCUUUACUUUAACAGATT |
| | antisense | UCUGUUAAAGUAAAGAUGCTG |
| *TLR8-4* | sense | CAAUAUUCGUUUUAACCAATT |
| | antisense | UUGGUUAAAACGAAUAUUGTC |

Briefly for each well of a 96 well plate, 10µl of siRNA duplexes (an equimolar mixture of the four described duplexes), diluted at 250nM in provided Buffer F, were combined with an equal amount of Viromer Blue, diluted in the same buffer at 500µM. SiRNA-transfection reagent complexes were allowed to form by incubation at room temperature for 15min. Cells were treated as describe above for human monocytes. Forty-eight hours post transfection cells were infected or treated as reported.

## T-cells differentiation studies

Experiments outlined in this section (i.e. monocytes: T-cells co-culture and conditioned media T-cell culture) were executed in collaboration with Jenny Gerhard, M.Sc. at the Department of Infectious Disease and Pulmonary Medicine of the Charité - Universitätmedizin Berlin.

For the monocyte:T cell co-cultures, monocytes were isolated by negative immunomagnetic selection and cultivated as described above. Cells were plated at a concentration of $4 \times 10^4$ cells/ml and stimulated as indicated for 1.5h in antibiotic-free medium. For co-culture experiments in Fig. 25 the following ligands, and concentrations, were used: CL075 (0.1, 0.5 and 1µg/ml), MPLA (0.1, 0.5 and 1µg/ml), and CpG (0.1, 1 and 2.5µM). Subsequently autologous naïve $CD4^+$ T cells were added to the stimulated monocytes at a monocyte to T cell

ratio of 2:1. Concomitantly, penicillin/streptomycin (1%) was added together with staphylococcal enterotoxin B (SEB) at a concentration of 1μg/ml. The co-cultured cells were kept for 5 days at 37°C, 5% $CO_2$ in a humidified incubator. At the end of the incubation period T cells were carefully harvested, washed twice in FACS buffer and analyzed by flow cytometry.

To investigate the role of soluble factors produced by stimulated APCs on T cell development and differentiation, supernatants collected from treated monocytes (conditioned media) was used to stimulate naïve $CD4^+$ T cells. As opposed to the procedure described above, no autologous monocytes were used in this experimental setup thus excluding the effects of cell-to-cell contacts. $CD4^+$ T cells were polyclonally activated by plate-bound anti-CD3 and soluble anti-CD28 antibodies. Briefly, cell culture 96-well plates were coated overnight at 4°C with anti-CD3 antibodies at a concentration of 4μg/ml and, after removal of the coating antibodies, T cells were subsequently added together with soluble anti-CD28 antibodies at a final concentration of 1μg/ml. Cells were subsequently cultivated in the presence supernatants collected from monocytes stimulated for 18h with the indicated bacteria or synthetic receptor ligands. In particular, APCs were previously stimulated with TLR ligands MPLA (1μg/ml), CL075 (3M002; 1μg/ml), LPS-EK Ultrapure (2μg/ml), Pam3CSK4 (200ng/ml), Poly(I:C) LMW (2μg/ml), ODN 2395 (5μM) or R848 (1μg/ml).

Human monocytes treated with siRNA (as described in detail in the relevant section) directed against TLR8 (and a corresponding negative control) for 48h, were treated with the indicates stimuli and supernatants collect after overnight incubation. The conditioned media were used for stimulation of total $CD4^+$ T cells as described above.

Conditioned media were filtered through a 0.22um pore size filter prior to their use. Conditioned media was used at 1:1 ratio with fresh T cell medium and cells were cultivated at a concentration of 2.5 x $10^5$ cells/ml for 5 days at 37°C, 5% $CO_2$. After 5 days, the cell culture supernatants were collected for further cytokine analysis by ELISA and the cells harvested for flow cytometry analysis of $CD4^+$ T-cell polarization.

## Flow cytometry and cell sorting

Flow cytometry analyses were performed on a BD FACSCanto II cytometer provided by the flow cytometry facility located at the Berlin-Brandenburg Centre for Regenerative Therapies (BCRT), Charité – Universitätmedizin Berlin.

Surface staining of CD4 was performed and cells analysed by flow cytometry after 5d of co/culture or incubation with conditioned supernatants. For this purpose, the T cells were

incubated with an antibody against human CD4 in the dark at 37°C for 60min on a shaking platform. Cells were then washed twice and resuspended in the appropriate volume of FACS buffer until measurement. Flow cytometry analysis of intracellular cytokines (i.e. IL-21) and transcription factors (i.e. BCL6) was performed to evaluate $T_{FH}$ polarization. On day 5 after incubation with APC-derived media or monocyte:T cells co-culture, cells were stimulated for 5 hours with 50ng/ml of PMA and 1ug/ml of Ionomycin. After 2.5h, the further secretion of cytokines was blocked by the addition of Brefeldin A and Monensin. Cells were subsequently washed twice in the appropriate buffer and fixed and permeabilized using Cytofix/Cytoperm Fixation and Permeabilization Solution according to manufacturer's guidelines. Fixed and permeabilized cells were then stained with antibodies against IL-21 (1:100) for 20min at room temperature. Staining for BCL6 was achieved by fixation and permeabilization using the Foxp3/Transcription Factor Staining Buffer Set according to manufacturer's instruction. Cells were incubated with anti-BCL6 antibodies (1:50) for 60 min at room temperature with shaking at 400rpm. Subsequently, the stained cells were washed twice in permeabilization buffer before being taken up in FACS buffer for analysis. An analogous protocol was used for flow cytometry analysis of porcine $CD4^+$ cells using the respective porcine reactive antibodies.

Surface markers of monocyte activation following stimulation were assessed by flow cytometry 18h after stimulation. Briefly, cells were harvested, washed in FACS buffer and stained in the same buffer for 30min at 4°C with an antibody mixture containing ICOSL (1:100), CD40 (1:200), CD80 (1:200), MHCII (1:200), and OX40L (1:500). Cells where then washed twice in the same buffer and resuspended in FACS buffer until analysis.

$CD115^+CD14^+CD16^-$ monocytes, used for gene array analysis, were sorted from PBMCs on a BD FACSAria II SORP cell at the core facility of the Berlin-Brandenburg Centre for Regenerative Therapies (BCRT), Charité – Universitätmedizin Berlin. Cell purity checks were performed and a purity of >97% was confirmed. Cells were cultured in the media and conditions described above. Porcine $CD14^+CD172^+$, $CD14^-CD172^+$, and $CD4^+$ cells were sorted by flow cytometry (BD FACSAria III) at the Department of Veterinary Medicine of the Freie Universität Berlin. Isolated cells were cultured in antibiotic-free RPMI1640 supplemented with 10% FCS, 1% glutamine, 1% HEPES buffer, 1% Non-Essential Amino Acids.
Data were analysed using FlowJo software (Treestar, San Carlos, CA).

## Animal experiments

### *Sus scrofa domestica*

Swine studies were conducted in accordance with the Danish Animal Welfare Act under approval and authorization issued by the Danish Animal Experiment Inspectorate. Rearing of animals, sacrifice and collection of samples were all performed at National Veterinary Institute, Technical University of Denmark, Lindholm, Denmark by Prof. Gregers Jungersen and Dr. Kristoffer Jarlov Jensen at the Department of Biotechnology and Biomedicine, Technical University of Denmark.

In total, 18 five-week-old pigs (Danish Landrace/Danish Yorkshire crossbreeds, paternal lineage Duroc) of both sexes were raised and purchased on a commercial farm (Bøgekærgård, Faxe, Denmark). The animals were stratified according to size (6.3 to 10.4kg, averaging 8.0kg) and sex, and assigned to the three indicated treatments groups. Vaccinations were performed subcutaneously via injection of 1ml in the right side of the neck as follows: 1) live *Salmonella enterica* serovar Typhimurium vaccine (ST; Salmoporc-STM Ch.-B. 022 07 15, IDT Biologika, Dessau-Roßlau, Germany) containing 3.32 x $10^8$ CFU per dose, administered according to the product instructions; 2) heat-inactivated (65°C for 90 minutes) reconstituted Salmoporc STM vaccine (HKST) using the same dose as in 1); 3) saline alone. The live vaccine was administered within 2h of reconstitution in the provided saline solution. Fourteen days after primary immunization, the same regimen was repeated as booster injections. Proper heat-killing of the vaccine preparation was confirmed by absence of bacterial growth on LB plates incubated at 37°C for 24h. Throughout the entire duration of the experiment, the animals belonging to the three treatment groups were mixed and housed in two adjoining boxes. One pig, belonging to the live vaccine group was euthanized on day 19 of the experiment due to severe umbilical hernia. This was unrelated to the vaccination procedure. One pig in the control group presented on day 0 of the experiment the hallmarks of a suspected pneumonia (i.e. fever, dyspnea and generalized fatigue) and was subsequently treated successfully with 160mg benzylpenicillin and 200mg dihydrostreptomycin (0.8ml Streptocillin Vet) over 3 consecutive days. It was, therefore, excluded from further analysis. For these reasons, five animals per group were ultimately included in the final analyses.

Animals were sacrificed according to Danish animal welfare regulations. Transverse sections of spleen were fixated in 10% neutral-buffered formalin (4% formaldehyde) for immunohistochemistry. Moreover, spleen tissues samples and prescapular lymph node sections (LN, *cervicales superficiales dorsalis* which is the draining lymph node from the injection site)

were homogenized using disposable scalpels and single-cell suspensions were isolated by forcing homogenized tissue samples through a cell strainer (70μm). Cell suspension were subsequently treated with erythrocyte lysis buffer for 5min at room temperature, washed twice with RPMI1640 and cultured in RPMI1640 supplemented with 10% FCS, 1% glutamine, 1% HEPES buffer, 1% Non-Essential Amino Acids. Cells were grown at 37°C, 5% $CO_2$ in a humidified incubator

For *in vitro* experiments reported in Fig. 11, spleens samples were collected from German Landrace pigs aged between 8 weeks and 1 year of both sexes and provided by the Department of Veterinary Medicine of the Freie Universität Berlin. Single cell suspensions were prepared, and cell sorted as described above. Stimulations were carried out as described for human cells.

*In vitro* experiments outlined in Figs. 30 to 32 were conducted on samples from German Landrace pigs aged between 8 weeks and 1 year of both sexes provided by the Institute of Immunology at the Friedrich-Loeffler-Institut, Federal Research Institute for Animal Health, Greifswald—Island of Riems, Germany. Single cell suspensions were prepared as described above. Total splenocytes (Fig. 32) were cultured in IMDM supplemented with 10% FCS and were stimulated with ST, HKST (at MOI: 0.1, 0.5, 1, 3), LPS (2μg/ml), CL075 (1μg/ml) or bacterial RNA (237ng) complexed with pLa (280 ng) in the presence of concanavalin A (2μg/ml). One hour after treatment, penicillin-streptomycin (1%) was added. After 4d, cells were re-stimulated with PMA (50ng/ml) and ionomycin (1μg/ml), harvested, washed twice in FACS buffer and analysed by flow cytometry as described. Live and dead cells were discriminated using Zombie Violet Fixable Viability Kit allowing for exclusion of dead cells from downstream analysis.

### *Salmo Salar*

All experiments and analyses involving Atlantic salmons were performed at the Department of Biology of the University of Bergen, Norway.

Ten non-vaccinated healthy Atlantic salmon (*Salmo salar*) used in this study, on average weighing 275g, were kept in the rearing facilities at the Industrial Laboratory at Bergen High Technology Centre, Bergen, Norway. A sea water temperature of 8°C was used throughout the study. Fish were quickly netted and scarified with a sharp blow to the head. Peripheral blood was collected from the *vena caudalis* using a syringe, transferred to heparinised containers and kept on ice until subsequent cell fractionation. Collected blood was diluted 1:2 in Leibowitz L-15+ medium supplemented with 2mM L-glutamine, 15mM HEPES, 10U/ml heparin and with 5% of a solution containing 0.41M NaCl, 0.33M $NaHCO_3$ and 0.66% D-glucose.

Peripheral blood leukocytes (PBL) were isolated on a discontinuous gradient as previously described[8]. Briefly, samples were loaded on gradients consisting of 4 ml of Percoll with a density of 1.075g/ml overlaid with 3ml of Percoll with a density of 1.060g/ml. After centrifugation at 400g for 40 minutes the leucocyte-containing fraction was collected from both the 1.075g/ml density layer and the 1.075–1.060g/ml interface of the gradient. The cell suspension was washed twice in HBSS medium and centrifuged at 200g for 10 minutes. Isolated PBL were diluted to 500µl in L-15+ medium and cell number, aggregation factor and percentage of viable cells were determined by use of a CASY cell counter. Cell concentration was adjusted to 7 x $10^6$ cells/ml in the same medium, based on counts of viable cells/ml. Three millilitres of cells suspension were plated in 6 well plates (2.1 x $10^7$cells/well in total) and incubated for 3h at 15°C. Non-adherent cells were subsequently removed with two repeated washes with 3ml of HBSS medium leaving only adherent monocytic cells. Monocytes, averaged at 4 x $10^5$cells/ well, were either left untreated or infected with viable or heat killed *E. coli* for 6h at 15°C. Pen/strep antibiotic were added 90 minutes post infection at a final 1% concentration. After incubation, supernatants (containing also residual non-adherent cells) were removed and cells were lysed in 600ul/well of lysis buffer for further RNA isolation. Total RNA was extracted using GeneElute Mammalian Total RNA miniprep kit and treated with DNaseI to ensure removal of genomic DNA. A NanoDrop ND-1000 UV-Vis spectrophotometer was used to quantify RNA concentration and quality by $OD_{260/280}$ and $OD_{260/230}$. cDNA was generated using qScript cDNA synthesis according to the manufacturer's instructions using random hexamers and a maximum of 1µg of total RNA per 20µl reaction. The synthesized cDNA samples were diluted to 2ng/µl in nuclease free water and stored at -20°C until analysis.

RT-qPCR was performed in a C1000 Thermal cycler with CFX96 real time system using SYBR Green JumpStart Taq ready mix kit for quantitative PCR and HPLC purified primers (Sigma-Aldrich). Two PCR primer sets of three potential reference genes (elongation factor 1α (*EF1α*), ribosomal protein S20 (*RPS20*) and β-actin) were tested to identify the most stable reference gene in the experimental conditions.

The primer sequences used were as follows:

| ***Gene name*** | ***Accession n.*** | ***Forward & reverse sequences (5'-3')*** |
|---|---|---|
| *il-12p40-b1* | HG917957 | TTGTCCCGCATCCATCTCACTGTAT<br>ATCTTGTCTGGCTTCACTATTTCTTG |
| *il-12p40-b2* | BT049762.1 | CATACCATCACCCAACAGAACG<br>GCCAAACACCTTATGTACTCATCTTC |

| | | |
|---|---|---|
| *il-12p40-c* | BT049114.1 | ATCAGAGATTGAAGGTGAAAGTAGAAC<br>GCTCCTCCTTTCCGTTGTCTTTA |
| *tnf-α1* | AY929385.1 | ACAAAATGGAGCCTCAACTGGA<br>AGTGTCAGCGGTAAGATTAGGATTG |
| *tnf-α2* | AY929386.1 | ACTCCATCGGGGATAATGCTAATCTA<br>GGACTCAGAATTACCATACTTTTGTTG |
| *il-6* | DQ866150.1 | TGCTGATAGGGCTGGTCAAAGA<br>AACACGCTTCCTCTCACTGGCA |
| *il-1β* | AY617117.1 | AACCGAGTTCAAGGACAAGGAC<br>GCCGACTCCAACTCCAACAC |
| *il-10* | EF165029 | ACTCCGCACATCCTTCTCCACCA<br>TCATGGCGGTGGGCAACACC |
| *rps20* | BTO60032 | ATCACCACCAGAAAGACACCCT<br>GAGGTGATGTGCTTGACAATCTCA |

The PCR reactions (25µl) contained 12.5µl of 2X SYBR green jumpstart Taq Ready Mix, 20ng of cDNA, 10mM of forward and reverse primers and 0.5µl nuclease-free water. The cycling conditions were 94°C for 5 min followed by 45 cycles of 94°C for 15 s and 60°C for 1 minute. Melting curve analyses were performed of each amplicon to ensure the specificity of the primers and a two-fold dilution curve cDNA was made for efficiency calculations. Negative controls without template and reactions without reverse transcriptase (-RT) were included for all master mixes. Calculated $C^t$-values for each target gene were normalized against the reference RPS20 using the Microsoft-Excel-based computer software Q-Gene. The mean normalized expression (MNE)-values were converted into n-folds of induction by defining the MNE value of the non-treated sample as 1.

Cytospin preparations of isolated leukocytes were prepared by centrifugation of 100µl of cell suspension of 1 x $10^6$ cells/ml at 1000rpm, medium acceleration, for 3min, using a Shandon Cytospin III cytocentrifuge. The cytospin preparations were air dried for 20h at room temperature prior to Colorrapid staining following product manual. Briefly cytospin slides are immersed 8-10 times in each of the three provided solutions (fixative, tetrabromfluorescin disodium salt, methylthionine chloride), rinsed with water and air-dried overnight before microscopy. Cytospin preparations were examined using a Zeiss Axioskop 2plus microscope.

### Anti-*S. enterica* IgG ELISA

Lysates of *S. enterica* serovar Typhimurium (Salmoporc-STM) in PBS were prepared by sonication (ten 30sec pulses with 30sec intervals on ice) and protein concentration adjusted at 3ug/ml. Lysates were used to coat 96-well microtiter plates overnight at 4°C and subsequently washed twice in ELISA wash buffer. Serum samples from immunized pig were serially diluted (12 dilutions) and incubated on the pre-coated plates overnight at 4°C. After washing plates were incubated with affinity-purified polyclonal goat antibody directed against swine IgG and labelled with HRP (1:250) for 1h at room temperature. Bound goat anti-pig IgG-HRP was visualized by the addition of TMB substrate and incubation at room temperature until colour development. The reaction was stopped by addition of 2N Sulfuric Acid. The titer of antibodies to *S. enterica* for each animal in the corresponding treatment group was visualized as absorbance readings at 450nm at a set serum samples dilution of 1:51.200.

### Immunohistochemistry

Immunohistochemical analyses and imaging were performed at the Institute of Veterinary pathology of the Freie Universität Berlin. Formalin fixed spleen sections were embedded in paraffin, cut into 2 μm sections for immunohistochemical analyses after dewaxing in xylene and rehydration in decreasing ethanol concentrations. Heat-mediated antigen retrieval, used for detection of PAX5 in spleen tissues, was performed in 10mM citric acid (pH 6.0), microwaved at 600W for 12min. Subsequently, tissue sections were incubated with a purified mouse monoclonal anti-PAX5 antibody overnight at 4°C. An immune-purified mouse antibody, at the same dilution, was used as a negative control. Slides were incubated with biotinylated, secondary goat anti-rabbit IgG antibody and subsequently with HRP-coupled streptavidin. Diaminobenzidine (DAB) was used as substrate for the HRP mediated colorimetric reaction. Slides were counterstained with haematoxylin, dehydrated through graded ethanol, cleared in xylene and covered with a cover slide. Whole slide images of spleen tissues were performed by Aperio CS2 digital pathology scanner (Leica Biosystems Imaging Inc.).

### Phylogenetic analyses

For the construction of maximum likelihood (ML) phylogenic trees, analysis was performed on the www.phylogeny.fr platform using default parameters (i.e. MUSCLE for multiple

sequence alignment, PhyML for phylogeny reconstruction and TreeDyn graphical rendering of the phylogenetic tree). Reported reliability of internal branches was assessed using the approximate likelihood ratio test (aLRT) test within the analysis standard pipeline. TLR8 amino acid sequences from nine vertebrate species were obtained from GenBank with accession numbers: NP_001029109 (*Bos taurus*), NP_619542 (*Homo sapiens*), NP_001123899 (*Macaca mulatta*), NP_573475. (*Mus musculus*), NP_001155165.1 (*Salmo salar*), ABM92444 (*Rattus norvegicus*), NP_999352 (*Sus scrofa*), AAW69376.1 (*Takifugu rubripes*) and XP_002933859.1 (*Xenopus tropicalis*).

The TLRs phylogenetic tree was constructed with the aforementioned procedure described for the TLR8 phylogenetic tree using protein sequences derived from *Homo sapiens* (hs), *Mus musculus* (mm), *Salmo salar* (ss) and *Sus scrofa* (sus), *Oncorhynchus mykiss* (om), *Rattus norvegicus* (rn) and *Danio rerio* (dr). The toll-like receptor (TLR1 to TLR10) sequences used were obtained from GenBank with accession numbers: TLR1 (hs: CAG38593.1; mm: AAG35062.1; sus: BAG12310.1; ss: AEE38252.1), TLR2 TLR1 (hs: AAH33756.1; mm: AAH14693.1; sus: ACZ82293.1; om: CCK73195.1), TLR3 (hs: ABC86910.1; mm: AAH99937.1; sus: ABB92547.1; ss: AKE14222.1), TLR4 (hs: AAF05316.1; mm: EDL31078.1; sus: AAW82895.1; dr: AAQ90475.1), TLR5 (hs: AAI09119.1; mm: AAI25248.1; sus: NP_001335700.1; ss: AEE38253.1), TLR6 (hs: BAA78631.1; mm: BAA78632.1; sus: NP_998925.2), TLR7 (hs: AAZ99026.1; mm: AAI32386.1; sus: ABQ52583.1; ss: CCX35457.1), TLR8 (listed above), TLR9 (hs: AAZ95520.1; mm: EDL21125.1; sus: AFO65060.1; ss: ABV59002.1), TLR10 (hs: AAY78491.1; rn: NP_001139507.1; sus: NP_001025705.1).

## Statistical analyses

The statistical tests and significance are described in the legend of the corresponding figure. Briefly, statistical analyses of in vitro experiments were performed using one-way ANOVA and correction for multiple comparisons or multiple t-test. Statistical differences between different groups were analyzed with a two-way ANOVA test. For all statistical analysis, a $P$ value of $< 0.05$ was considered statistically significant. Calculations and visualization of the data were performed using GraphPad Prism 6 Software (GraphPad Software).

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# 3 RESULTS

## 3.1 Sensing of bacterial viability modifies cytokine responses of APC

As extensively described in the introduction, the role of *vita*-PAMPs was initially characterized in mice with the identification of prokaryotic messenger RNA as the first member of this novel class of pathogen associated molecular patterns[99]. However, their contribution to human immunity, and the identity of *vita*-PAMPs and their receptors relevant to human immunity has remained unknown.

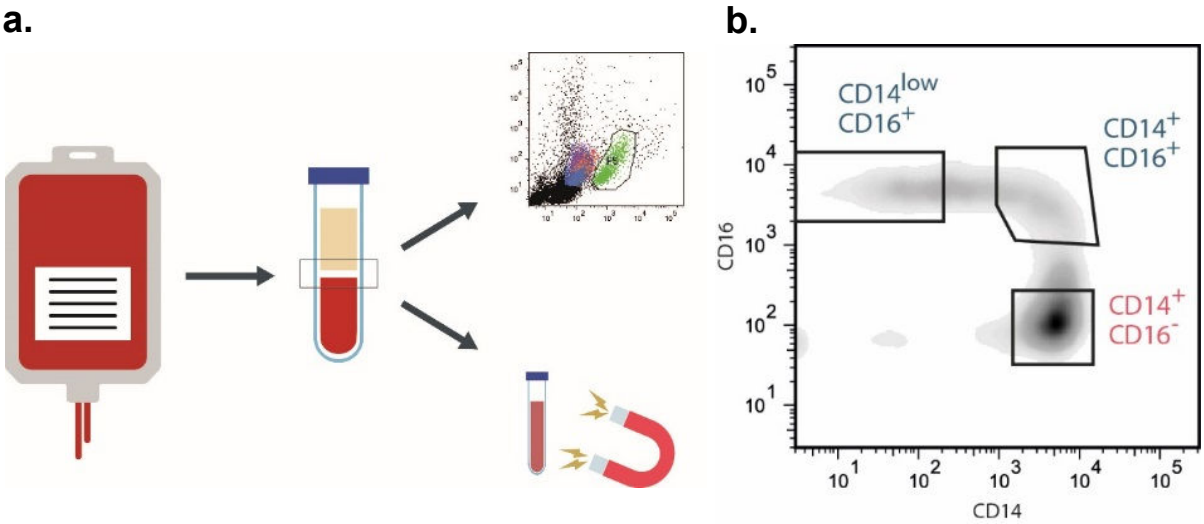


***Figure 2. Heterogeneity of human monocytes.*** ***(a)*** Schematic representation of the two complementary strategies used for the isolation of classical ($CD14^+CD16^-$) monocytes. Monocytes were purified either by immunomagnetic negative selection or by FACS from PBMC isolated by density gradient. ***(b)*** Representative FACS plot of the sorting strategy used for the isolation of human classical monocytes and their separation from non-classical ($CD14^{low}CD16^+$) and intermediate ($CD14^+CD16^+$) subpopulations.

In order to characterize the innate immune response triggered by recognition of bacterial

viability in humans, we used classical $CD14^{+}CD16^{-}$ monocytes as APCs. Monocytes were either isolated by *Fluorescence-activated cell sorting* (FACS) or purified via gradient isolation and subsequent immunomagnetic negative isolation from total PBMCs isolated from heathy donors (***Fig. 2***). Monocytes were chosen as representative APC due to their abundance (between 5 and 10% of total circulating PBMCs[188]) and their emerging role as key players in inflammation, infection and antigen presentation[157, 189]. Three distinct population of monocytes can be identified in humans on the basis of CD14 (LPS co-receptor) and CD16 (FCγIII receptor) expression: classical ($CD14^{+}CD16^{-}$), non-classical ($CD14^{low}CD16^{+}$) and intermediate ($CD14^{+}CD16^{+}$)[190]. Classical monocytes, as the most abundant subclass of monocytes, accounting for about 80% of total monocytes, were chosen due to their reported high phagocytic ability[191, 192] making them ideal candidates to study the processes of bacterial recognition. In order to systematically study the selective responses of human APCs to viable bacteria, we compared the transcriptional response induced by stimulation with viable *Escherichia coli* (EC), the same infection dose of *heat-killed Escherichia coli* (HKEC) or medium alone (Ctrl). Avirulent

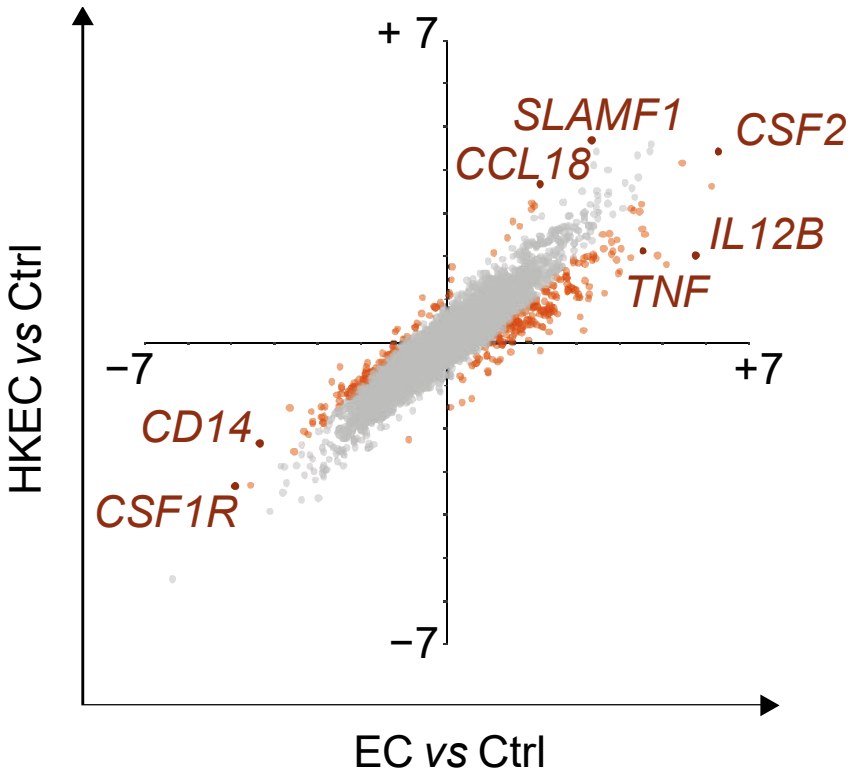


***Figure 3. Detection of viable bacteria induces transcriptional remodelling in human monocytes.*** Human $CD14^{+}CD16^{-}$ monocytes (n=4 donors) were stimulated with either medium (ctrl), EC or HKEC for 6 hours and subjected to genome-wide transcriptional analysis. Scatter plot in which the mean signal log ratio (SLR) for each analysed gene in EC-treated cells relative to control cells (EC vs Ctrl) are plotted against HKEC-treated relative to that of control cells (HKEC vs Ctrl). Orange circles indicate genes with an SLR difference >2 in EC-treated cells versus HKEC treated cells. Selected immune relevant genes are highlighted (n=4 donors).

thymidine auxotroph (*thyA*$^{-}$, replication defective) *Escherichia coli* K12-DH5α, a commonly used laboratory strain, was intentionally used in these studies to selectively investigate the effects of bacterial 'viability recognition' while excluding cofounding factors such as virulence and active replication: the bacteria, indeed, can only replicate, in the presence of selection antibiotics (trimethoprim), in culture media supplemented with thymidine. Moreover, to focus on the initial steps of bacterial phagocytosis and recognition, antibiotics were added to the infected cells ninety minutes post infection to inhibit any residual bacterial growth.

A genome-wide transcriptional analysis revealed that recognition of bacteria induced very robust transcriptional profiles in human monocytes, regardless of the viability of the bacterial stimulus (***Fig. 3***). The reason for this highly comparable response to both EC and HKEC lies in the high

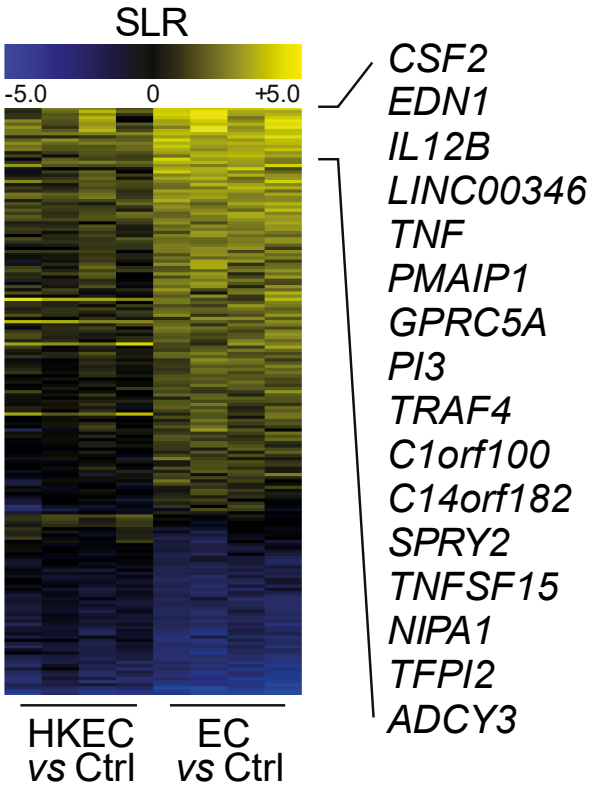


***Figure 4. A set of genes is specifically regulated in response to bacterial 'viability recognition'.*** Heat-map of the 193 regulated genes with an expression SLR fold change >2 in 'EC vs Ctrl' compared to 'HKEC vs Ctrl' as described in Fig. 3. The sixteen most strongly regulated are reported on the right (n=4 donors).

PAMP contents in both bacterial preparations, as both stimuli, indeed, share an abundance of components known to induce activation of the innate immune pathways (i.e. LPS) through pattern recognition receptors highly expressed in human monocytes.

However, a restricted set of 193 genes was differentially transcribed (for which *signal log ratio*, SLR, difference >2 in EC-treated cells versus HKEC-treated counterpart) in response to live compared to dead bacteria (***Fig. 4***). This transcriptional signature included genes encoding

proinflammatory cytokines (i.e. *TNF* and *IL12B*), an uncharacterized long non coding RNA (*LINC00346*), *G protein-coupled receptor C5A* (*GPRC5*A)[193], the adapter protein *TNF receptor associated factor 4* (*TRAF4*[194]) and also IFN-inducible genes such as *Interferon Induced Protein With Tetratricopeptide Repeats 2* (*IFIT2*; Fig. 3, Fig. 4 and ***Table 2***).

These data provide evidence that human monocytes are clearly able to distinguish between live and dead bacteria and respond with a defined, and reproducible, remodelling of their transcriptional profile.

*Table 2.* Comprehensive list of the 193 genes, which show differential regulation in response to live versus dead Gram-negative bacteria. The sixteen most strongly regulated genes are highlighted in the heat map in Fig. 4.

| | gene name | gene ID | | gene name | gene ID | | gene name | gene ID | | gene name | gene ID |
|---|---|---|---|---|---|---|---|---|---|---|---|
| 1 | CSF2 | 1437 | **32** | NEDD4L | 23327 | **63** | RRAD | 6236 | **94** | NRARP | 441478 |
| 2 | EDN1 | 1906 | **33** | ADRB2 | 154 | **64** | TAGAP | 117289 | **95** | TEX14 | 56155 |
| 3 | IL12B | 3593 | **34** | TNFSF9 | 8744 | **65** | TBC1D7 | 51256 | **96** | RHOB | 388 |
| 4 | LINC00346 | 283487 | **35** | C12orf50 | 160419 | **66** | KLF4 | 9314 | **97** | CD200 | 4345 |
| 5 | TNF | 7124 | **36** | ADTRP | 84830 | **67** | NA | 255352 | **98** | TCF7L2 | 6934 |
| 6 | PMAIP1 | 5366 | **37** | PSMD5 | 5711 | **68** | RGS1 | 5996 | **99** | ADORA2B | 136 |
| 7 | GPRC5A | 9052 | **38** | HIVEP3 | 59269 | **69** | CCL18 | 6362 | **100** | SNORD12C | 26765 |
| 8 | PI3 | 5266 | **39** | EXT1 | 2131 | **70** | GCLC | 2729 | **101** | TICAM1 | 148022 |
| 9 | TRAF4 | 9618 | **40** | DENND4A | 10260 | **71** | IGSF3 | 3321 | **102** | CKS2 | 1164 |
| 10 | C1orf100 | 200159 | **41** | GRAMD3 | 65983 | **72** | EGR3 | 1960 | **103** | RASGEF1B | 153020 |
| 11 | C14orf182 | 283551 | **42** | LINC00158 | 54072 | **73** | GPR132 | 29933 | **104** | KLHL21 | 9903 |
| 12 | SPRY2 | 10253 | **43** | NEU4 | 129807 | **74** | P2RX7 | 5027 | **105** | KIAA1199 | 57214 |
| 13 | TNFSF15 | 9966 | **44** | PELO | 53918 | **75** | HIST1H2AH | 85235 | **106** | IL18 | 3606 |
| 14 | NIPA1 | 123606 | **45** | RAB33A | 9363 | **76** | GGT5 | 2687 | **107** | HIST1H2BG | 8339 |
| 15 | TFPI2 | 7980 | **46** | HECW2 | 57520 | **77** | TMEM217 | 221468 | **108** | CNST | 163882 |
| 16 | ADCY3 | 109 | **47** | ABCA5 | 23461 | **78** | INPP5F | 22876 | **109** | CD109 | 135228 |
| 17 | TNC | 3371 | **48** | RASD1 | 51655 | **79** | ACOX3 | 8310 | **110** | HIST1H3H | 8357 |
| 18 | SPINK7 | 84651 | **49** | EGR1 | 1958 | **80** | NLRP3 | 114548 | **111** | TJP1 | 7082 |
| 19 | CLEC2D | 29121 | **50** | C5orf62 | 85027 | **81** | NFKBIZ | 64332 | **112** | TCTEX1D4 | 343521 |
| 20 | PHF19 | 26147 | **51** | CARD17 | 440068 | **82** | CDKN2A | 1029 | **113** | RAB3IP | 117177 |
| 21 | IFIT2 | 3433 | **52** | RNU11 | 26824 | **83** | TNFRSF21 | 27242 | **114** | FOSB | 2354 |
| 22 | VTRNA1-3 | 56662 | **53** | PRC1 | 9055 | **84** | CHST7 | 56548 | **115** | SCUBE2 | 57758 |
| 23 | FXYD6 | 53826 | **54** | CYB5D1 | 124637 | **85** | ITPKC | 80271 | **116** | IL28RA | 163702 |
| 24 | HSPA2 | 3306 | **55** | ID1 | 3397 | **86** | MIR17HG | 407975 | **117** | TGFB2 | 7042 |
| 25 | IRF8 | 3394 | **56** | TLCD1 | 116238 | **87** | ADNP2 | 22850 | **118** | DTX4 | 23220 |
| 26 | EPS8 | 2059 | **57** | HIST1H3A | 8350 | **88** | HIST1H3I | 8354 | **119** | JUN | 3725 |
| 27 | DUSP5 | 1847 | **58** | EIF4E | 1977 | **89** | GADD45G | 10912 | **120** | SNORA57 | 692158 |
| 28 | CYP19A1 | 1588 | **59** | THAP4 | 51078 | **90** | LTA | 4049 | **121** | SNORD104 | 692227 |
| 29 | DCSTAMP | 81501 | **60** | FAM126A | 84668 | **91** | SNORD74 | 619498 | **122** | LIF | 3976 |
| 30 | CCNA1 | 8900 | **61** | TCTEX1D2 | 255758 | **92** | PHLDA2 | 7262 | **123** | SNORD116-6 | 100033418 |
| 31 | ATP6V0D2 | 245972 | **62** | USP53 | 54532 | **93** | CLCF1 | 23529 | **124** | HNF1B | 6928 |

| | gene name | gene ID | | gene name | gene ID | | gene name | gene ID | | gene name | gene ID |
|---|---|---|---|---|---|---|---|---|---|---|---|
| 125 | JAZF1 | 221895 | **142** | P2RY2 | 5029 | **159** | MRPL30 | 51263 | **177** | SLC9A6 | 10479 |
| 126 | LIPA | 3988 | **143** | SEMA4B | 10509 | **160** | C3AR1 | 719 | **178** | ACSS2 | 55902 |
| 127 | SGMS2 | 166929 | **144** | SLC16A10 | 117247 | **161** | IRAK4 | 51135 | **179** | SEMA4A | 64218 |
| 128 | CD226 | 10666 | **145** | GCA | 25801 | **162** | GNAI2 | 2771 | **180** | GPR141 | 353345 |
| 129 | TMCC3 | 57458 | **146** | PDE4A | 5141 | **163** | HS3ST1 | 9957 | **181** | DPYD | 1806 |
| 130 | CDCA4 | 55038 | **147** | ACP2 | 53 | **164** | PNPLA6 | 10908 | **182** | PXK | 54899 |
| 131 | NFKBID | 84807 | **148** | C12orf5 | 57103 | **165** | PIGM | 93183 | **183** | NPL | 80896 |
| 132 | MYC | 4609 | **149** | BAK1 | 578 | **166** | MAPK14 | 1432 | **184** | LPCAT3 | 10162 |
| 133 | HIST1H2BD | 3017 | **150** | TUBG1 | 7283 | **167** | USF1 | 7391 | **185** | CLEC4A | 50856 |
| 134 | ST8SIA4 | 7903 | **151** | IL1RAP | 3556 | **169** | ZYG11B | 79699 | **186** | ENG | 2022 |
| 135 | HIST1H3D | 8351 | **152** | CTSH | 1512 | **170** | PPIP5K2 | 23262 | **187** | NAGA | 4668 |
| 136 | SLC9A7 | 84679 | **153** | RIOK2 | 55781 | **171** | CPPED1 | 55313 | **188** | FAM198B | 51313 |
| 137 | MARCH3 | 115123 | **154** | GPR31 | 2853 | **172** | PLD2 | 5338 | **189** | NCKAP1L | 3071 |
| 138 | GPD2 | 2820 | **155** | DPP3 | 10072 | **173** | PTPRC | 5788 | **190** | VCAN | 1462 |
| 139 | MRPL9 | 65005 | **156** | NSL1 | 25936 | **174** | TBC1D2B | 23102 | **191** | CD14 | 929 |
| 140 | ZBTB38 | 253461 | **157** | TXNL4A | 10907 | **175** | CCDC109B | 55013 | **192** | TGFBI | 7045 |
| 141 | MB21D1 | 115004 | **158** | KIAA1467 | 57613 | **176** | UCP2 | 7351 | **193** | CSF1R | 1436 |

## 3.2 Recognition of live bacteria induces a specific cytokine secretion pattern

Analysis of secreted cytokines by monocytes, stimulated for 18 hours with either viable or heat-killed bacteria confirmed that the observed transcriptional response translated into an altered cytokine profile. A set of proinflammatory cytokines was selectively induced in response to live but not dead bacteria, including IL-12p40 and IL-12p70 (the biologically active form of IL-12, a heterodimer of disulphide-bound subunits designated p40 and p35) and TNF (***Fig. 5***).

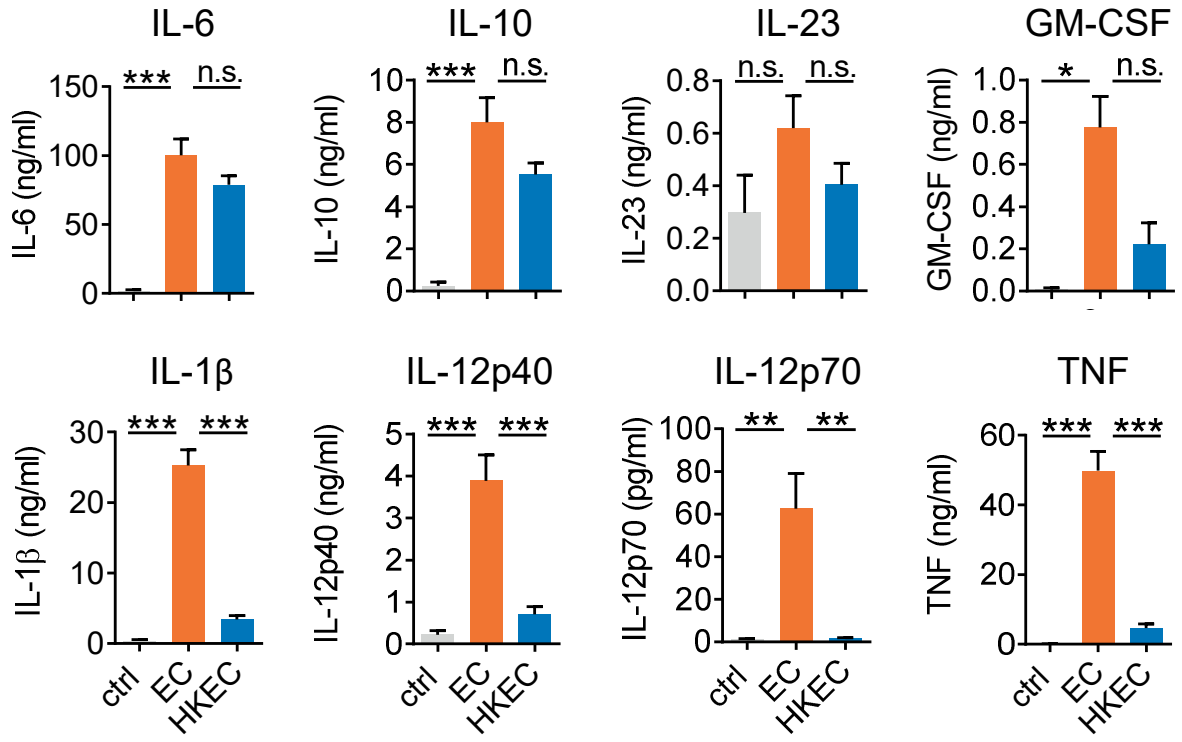


***Figure 5. Cytokine secretion of human APCs in response to viable bacteria***. Cytokine secretion measured in the culture supernatants of human APCs left untreated (ctrl), stimulated with live *Escherichia coli thyA*$^{-}$ (EC) or heat-killed bacteria of the same strain (HKEC) for 18h. Data are representative of three to six independent experiments (one per donor): n=3 (GM-CSF), n=4 (IL-23), n=5 (TNF, IL-6, IL-10 and IL-12p40) or n=6 (IL-1B and IL-12p70). Bars indicate mean + SEM. ** $p \leq 0.01$, *** $p \leq 0.001$; n.s. =non-significant (one-way analysis of variance (ANOVA) with post-hoc correction for multiple comparisons).

Other cytokines such as IL-6, IL-10, IL-23 and GM-CSF were produced in response to bacterial stimulation, but independently of bacterial viability (Fig. 5). These results were unexpected, given the previously published observations with murine BMDMs, which were shown to secrete large amount of IL-12 and TNF in response to both live and killed bacteria or purified bacterial cell wall components[99]. In contrast, human APCs secrete proinflammatory cytokines exclusively upon infection with viable bacteria.

In agreement with results in the murine system, IL-1β production was restricted to stimulation with viable bacteria in human APCs[99] (Fig. 5). IL-1β production is regulated on

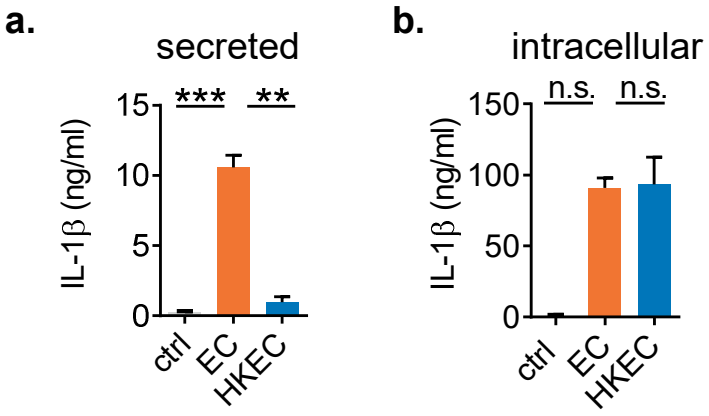


***Figure 6. Translation and secretion of IL-1β in response to bacterial viability.*** ***(a)*** IL-1β secretion measured in the culture supernatants of human APCs left untreated (ctrl), stimulated with EC or HKEC for 18h. ***(b)*** Release of intracellular pro-IL-1β measured in cells disrupted with repeated (5x) freezing-towing cycles 18 h post infection (n=4 independent experiments with one donor). Bars are mean + SEM. ** p≤0.01, *** p≤0.001, n.s. =non-significant (one-way ANOVA with post-hoc correction for multiple comparisons).

multiple levels[195]. The absence of differences in *pro-IL1B* transcription suggests a regulation at posttranscriptional/posttranslational level, namely through the regulation of the inflammasome dependent processing[99]. Indeed, data generated in our laboratory reveals a selective activation of the NLRP3 inflammasome in response to live, but not killed bacteria (in collaboration with Moritz Pfeiffer and Elisa Helbig, in the group of Prof. Dr. Sander). Further supporting this result, intracellular levels of non-cleaved pro-form of IL-1β were similar after stimulation with EC or HKEC, whereas only live EC induce the secretion of bioactive, cleaved IL-1β (***Fig. 6***)

The observed differences at the transcriptional and cytokine secretion level were caused by a

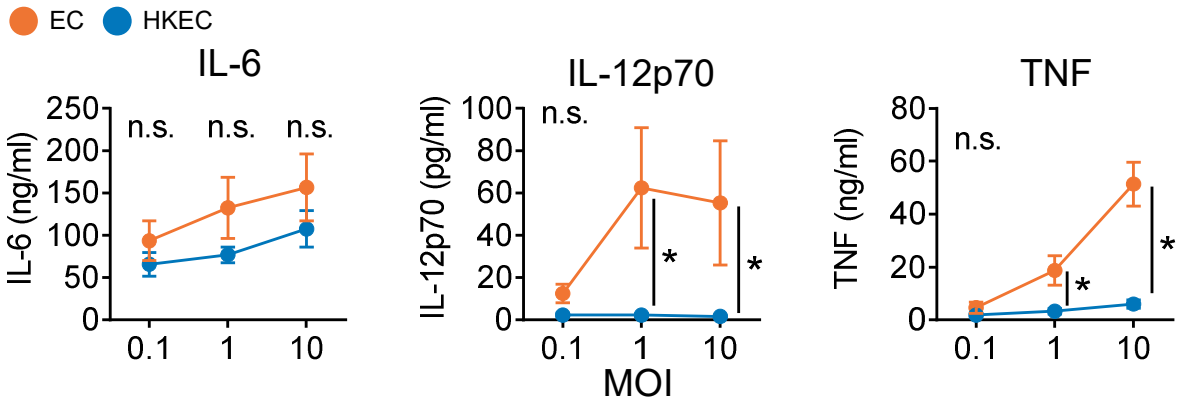


***Figure 7. Cytokine secretion from APCs stimulated with increasing multiplicity of infection (MOI).*** Human monocytes where stimulated with EC (orange) or HKEC (blue) as in Fig. 5 with different MOI. Concentrations of secreted IL-6, TNF, and IL-12p70 were measured 18h post treatment (n=4 donors). Error bars are mean ± SEM. * p≤0.05; n.s.=non-significant (Multiple t-test).

qualitative rather than a quantitative difference between EC and HKEC stimulation, since

increasing doses of heat-killed bacteria did not restore the production of IL-12 or TNF (***Fig.* 7**). On the other hand, IL-6 secretion at varying multiplicities of infection (MOI) was independent of bacterial viability (Fig. 7), hence underscoring that HKEC are still recognized as foreign by the immune system.

## 3.3 Presence of *vita*-PAMPs in different bacterial species

To test if the recognition of viable bacteria was restricted to specific bacterial species or Gram staining characteristics, analogous infection experiments were performed with other avirulent bacterial strains, namely Gram-positive *Bacillus subtilis* and *Bacillus Calmette-Guerin* (BCG),

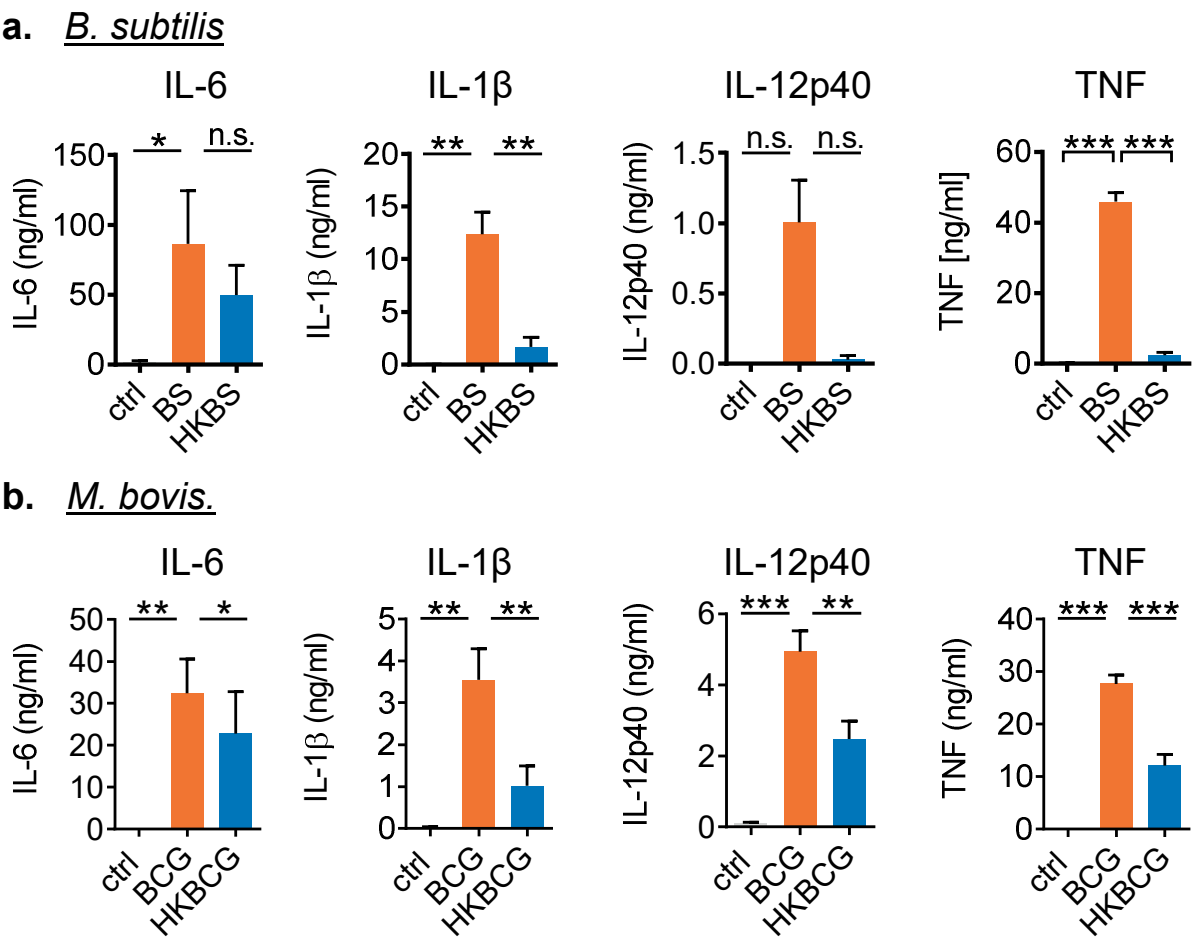


***Figure 8. Recognition of bacterial viability is independent from the bacterial species.*** Cytokine secretion from APCs left untreated (ctrl) or stimulated for 18h with ***(a)*** live or heat-killed *Bacillus subtilis* (BS and HKBS respectively, n=3-5 experiments, one per donor), (***b)*** live or heat-killed *Mycobacterium bovis* strain BCG (BCG and HKBCG respectively, n=4 experiments, one per donor). Bars are mean + SEM. ⋆ p≤0.05, ⋆⋆ p≤0.01, ⋆⋆⋆ p≤0.001; n.s.=non-significant (one-way ANOVA with post-hoc correction for multiple comparisons).

an attenuated strain of *Mycobacterium bovis* and widely used vaccine against tuberculosis. These particular strains were selected due to their severe attenuation, species-specific features and, in

case of BCG, routine use as vaccine and immunoactive agent. Despite their diverse morphology and infection behaviours, both strains elicited similar cytokine patterns ***(Fig. 8a, b)*** characterized by secretion of IL-12, TNF and IL-1β only in response to viable bacteria.

Hence, recognition of bacterial viability by human APCs occurs independently of bacterial dose and species-specific features and, thus, appear to be a central motive in innate immunity.

## 3.4 APC maturation occurs independently of bacterial viability

APCs promote T cell priming and differentiation via presentation of antigens, engagement of co-stimulatory signals and the secretion of cytokines. Upregulation of MHC-II molecules and co-stimulatory markers on APCs is often referred to as *maturation*. While the secretion of cytokines is markedly skewed in response to live bacteria (Fig. 5), both viable heat-killed bacteria similarly induce up-regulation of several maturation markers instrumental in orchestrating an efficient adaptive immune response, including MHC-II, CD40, CD80, ICOS ligand and OX40 ligand[170] ***(Fig. 9)***.

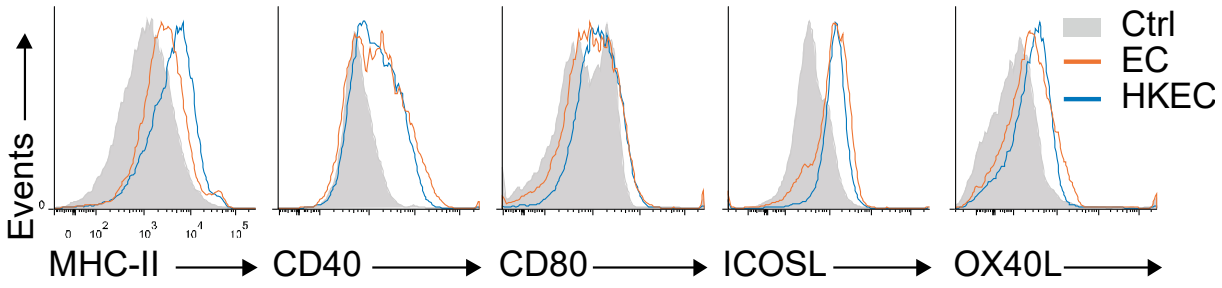


***Figure 9. Expression of activation markers and costimulatory molecules upon 'viability sensing'.*** APCs were treated as in Fig. 5 and surface expression of the indicated markers was measured by flow cytometry at 18h post infection (n=5 donors).

## 3.5 Evolutionary conservation of 'viability recognition'

Detection of microbial viability by the innate immune system seems to be applicable across a wide range of bacterial species. Moreover, it regulates central components of the innate immune response, constituting a critical innate immune checkpoint to scale the infectious threat level posed by a given microbial encounter[3]. Consequently, we next sought to investigate the evolutionary conservation of 'viability sensing' on the host side.

As previously described, murine innate immune cells actively distinguish between living and dead microbes via the selective sensing of bacterial mRNA, a so-called *vita*-PAMP, which leads to the activation of the NLRP3 inflammasome and secretion of IL-1β[99]. In contrast,

transcriptional responses, including TNF and IL-12 production, are largely independent of *vita*-PAMPs in murine APC.

To study the evolutionary conservation of this process beyond human and laboratory mice, we choose to examine two very distant species, which in the past years have gained considerable interest: outbred farm swine, *Sus scrofa domestica*, and the Atlantic salmon, *Salmo salar*. The former constitutes an appealing model for pharmaceutical and infection biology studies due to well-known physiological similarities with humans[196, 197]. Whereas interest in the immune system development and functions of fish, and salmon in particular, is fuelled by evolutionary studies[198-200], as well as the growing demand for effective vaccines for the rapidly growing fish farming industry.

### *Sus scrofa domestica*

Spleen samples were obtained from eight-weeks to one-year old domestic farm pigs,

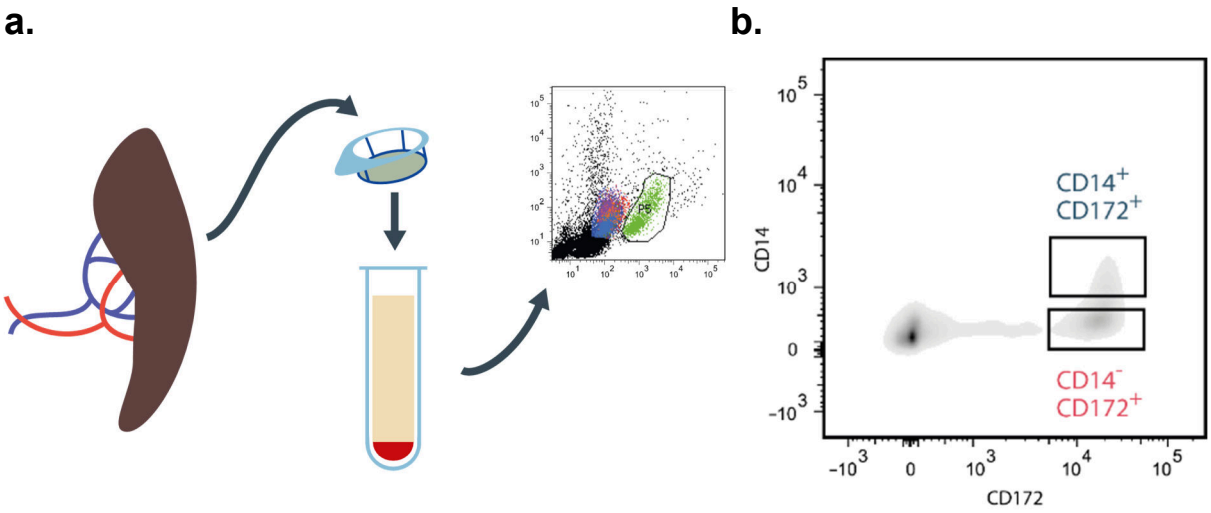


***Figure 10. Isolation of porcine monocytes and dendritic cells. (a)*** Schematic of the methodology used to process porcine spleen sections to obtain first a splenocyte suspension and, subsequently, highly purified cell populations via flow cytometry-based sorting. ***(b)*** Representative FACS plot of the sorting strategy used for the isolation of porcine monocytes ($CD14^+CD172^+$) and dendritic cells ($CD14^-CD172^+$) from the spleen of pigs.

homogenized and, subsequently, porcine monocytes (defined as $lin^-CD14^+CD172^+$ cells) and dendritic cells (DC, defined as $lin^-CD14^-CD172^+$ cells)[201] were sorted by flow cytometry from total splenocytes preparations (***Fig. 10***). Monocytes and DC were stimulated with either live or heat-killed *ThyA*$^-$ *E. coli* (EC and HKEC) or with an attenuated, adenine- and histidine auxotrophic vaccine strain of *Salmonella enterica* serovar Typhimurium[202], either live or heat-killed (ST and HKST respectively). Similar to human APC, porcine monocytes and DC produced large amounts of IL-12 selectively in response to living bacteria (EC or ST), but not

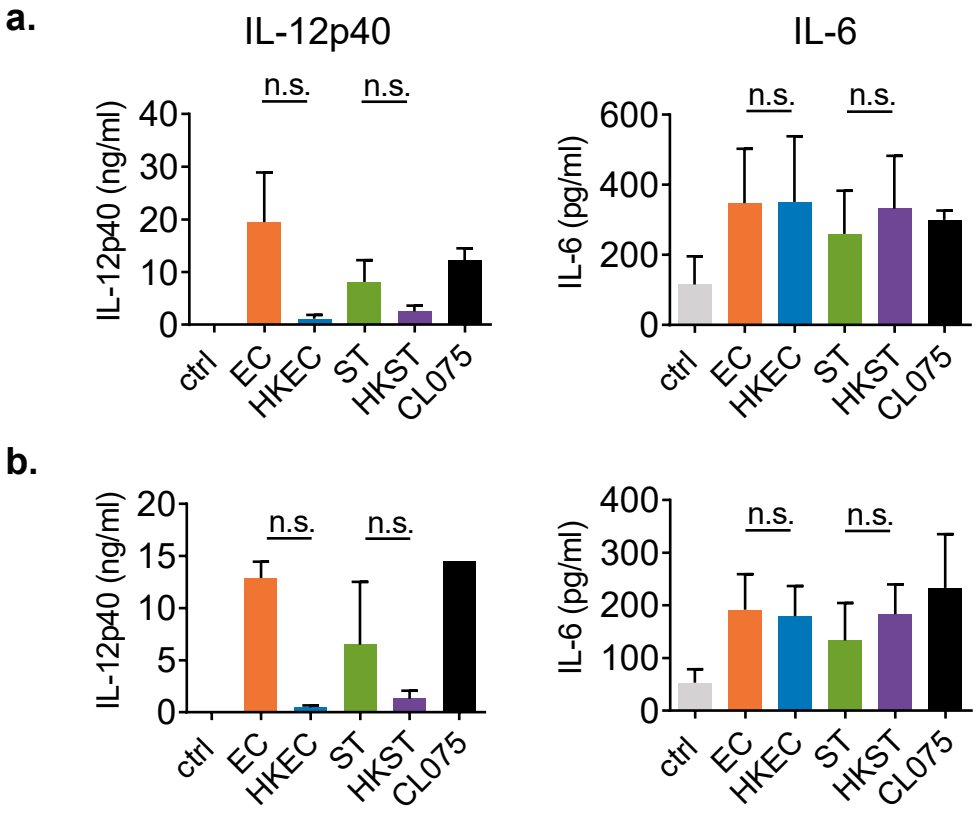


***Figure 11. Response to microbial viability in porcine APCs.*** Porcine monocytes ***(a)*** and dendritic cells ***(b)*** isolated from pig spleens by FACS were stimulated either with culture medium (ctrl), EC, HKEC, live attenuated *S. enterica* serovar Typhimurium (ST) or heat-killed ST (HKST) at a MOI of 10. IL-12p40 and IL-6 secretion in the culture supernatants was monitored by multiplex bead array at 24h post treatment (n=3 pigs for IL-6 and n=2 pigs for IL-12p40). Bars are mean + SEM. n.s.=non-significant (one-way ANOVA with post-hoc correction for multiple comparisons).

in response to their respective killed counterparts (HKEC or HKST). Contrariwise, IL-6 was secreted regardless of bacterial viability (***Fig. 11***). Despite being constantly recorded, induction of IL-12 selectively in response to live EC and ST, compared to their killed counterparts, did not reach statistical testing significance. This was due, most probably, to the limited sample size, and its high inter-experimental and inter-sample variability (in terms of sex and age of the individual animals). Both monocytes and dendric cells were also highly responsive to TLR8 synthetic ligand CL075

### *Salmo salar*

Leukocytes were isolated from peripheral blood of Atlantic salmon by discontinuous gradient centrifugation and monocytes were subsequently isolated by adherence (***Fig. 12***). Adherent cells

have been previously characterized as monocyte-/macrophage like cells[203]. Hence, adherent cells were stimulated with either control media, viable *E. coli* or heat-killed *E. coli*.

Interestingly also in salmon, monocytes/macrophages clearly discriminated bacteria according to their viability status as evidenced by the selective induction of proinflammatory cytokines TNF-α and IL-1β only in response to living bacteria, while IL-6 and the regulatory cytokine IL-10 are expressed in response to either bacterial stimulus (***Fig. 13***).

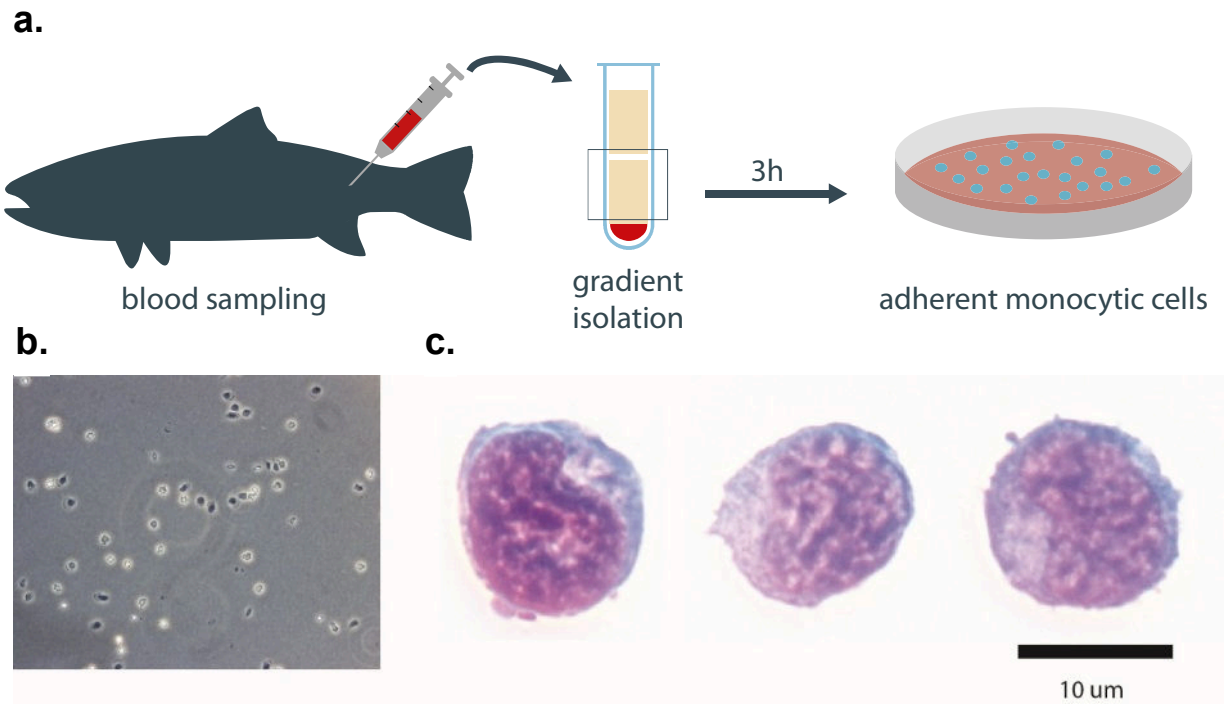


***Figure 12. Monocyte/macrophage-like cells isolated from Atlantic salmon.*** ***(a)*** Schematic depiction of the isolation strategy used to collect salmon monocytes. Leukocytes were isolated from blood sampled from the posterior *vena caudalis* of salmons by gradient centrifugation, plated and non-adherent cells were removed after 3h incubation. ***(b)*** Inverted microscopy pictures of adherent monocytic cells (20x magnification). ***(c)*** Cytospin preparation of representative cells. Pictures are captured with a 63x objective. Scale bar: 10 μm.

Three paralogs of IL-12p40 are present in the salmon genome, IL-12p40-b1, IL-12p40-b2 and IL-12p40-c respectively[204], which most likely arose during the four rounds (4R) of whole genome duplication (WGD) experienced by the salmonid lineage compared to other vertebrates[205]. Of the three identified IL-12p40 genes two are induced specifically in response to infection with live bacteria, while the third isoform does not show a viability-dependent pattern of expression (Fig. 13). This differential modulation reflects what has been previously observed with the three IL-12 paralogs in response to PAMP[204] stimulation and viral infection[206]. Collectively, these results indicate that the mechanism of microbial 'viability recognition' is likely largely conserved and a general feature of the vertebrate innate immune system being present even in teleost fishes, such as salmon, despite approximately 400 to 450 million years of

evolution separating them from mammals[207, 208] and the differing environmental niches occupied. Interestingly, also in *Drosophila melanogaster*, a negative regulation of the immune response in the presence of dead bacteria has been recently identified and shown to enable a response tailored to the threat level[209].

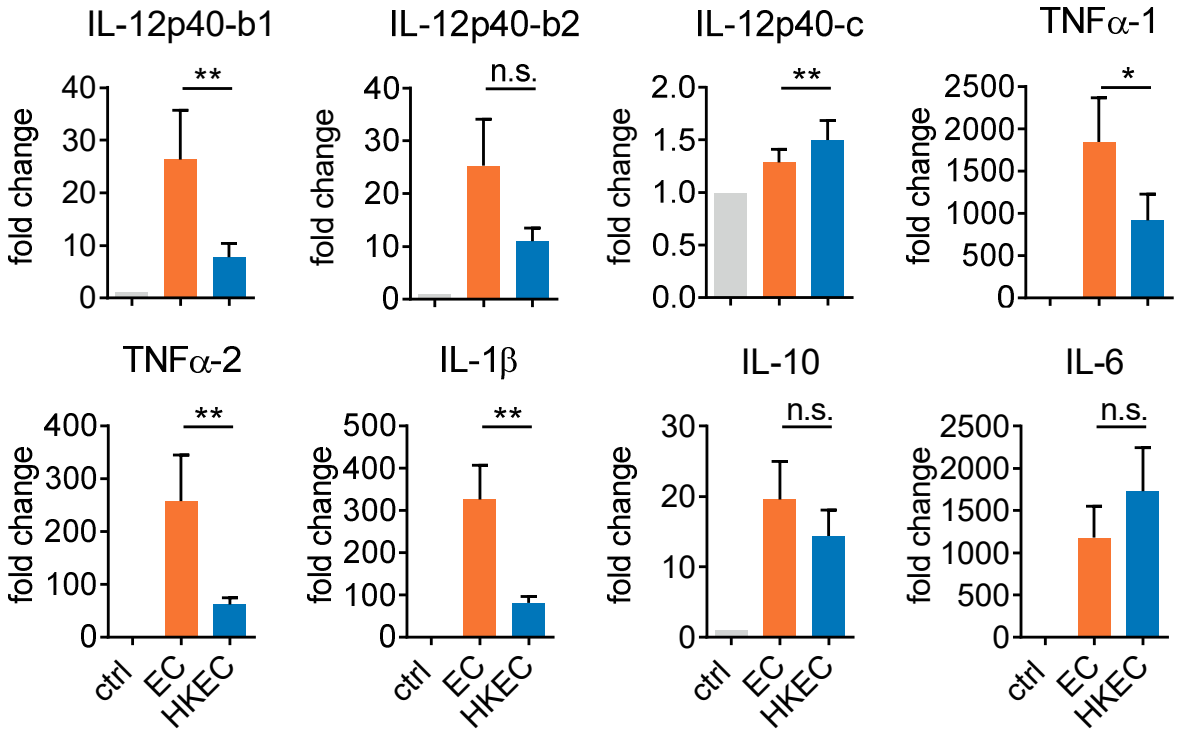


***Figure 13. APCs from Salmo salar discriminate live and dead bacteria.*** Salmon monocytes were either left untreated (ctrl) or infected with EC or HKEC at a MOI of 10. Expression of the indicated genes was measured 6 hours post infection via *quantitative reverse transcription polymerase chain reaction* (RT-qPCR). Expression values are shown as fold induction normalized to the reference gene ribosomal protein S20 (n=10 individual fishes). Bars are mean + SEM. ⋆ $p \leq 0.01$, ⋆⋆ $p \leq 0.01$; n.s.=non-significant (Student's t-test and Wilcoxon's correction).

## 3.6 Involvement of TLRs in the recognition of bacterial viability

Based on our findings showing that APCs specifically detect bacterial viability, we next proceeded to investigate the identity of the sensing receptor(s) and the nature of the ligands, i.e. the *vita*-PAMP(s). Given the critical role of PAMPs[13], we supplemented a heat-killed preparation of bacteria with various known ligands of TLRs to test if the activation of individual TLR could restore the specific cytokine responses to living bacteria. The following TLR ligands were included in the screen: synthetic triacylated lipoprotein Pam3CSK4 (TLR2), polyinosine-polycytidylic acid (Poly(I:C), TLR3), lipopolysaccharide from *E. coli* K12, (LPS-EK, TLR4), thiazoquinoline compound CL075 (TLR7/8), CpG oligonucleotide ODN 2395 (TLR9).

Among all the ligands tested only the base analogue CL075, ligand of single stranded RNA receptors TLR7 and 8 was able to restore the secretion of IL-12 and TNF to an extent

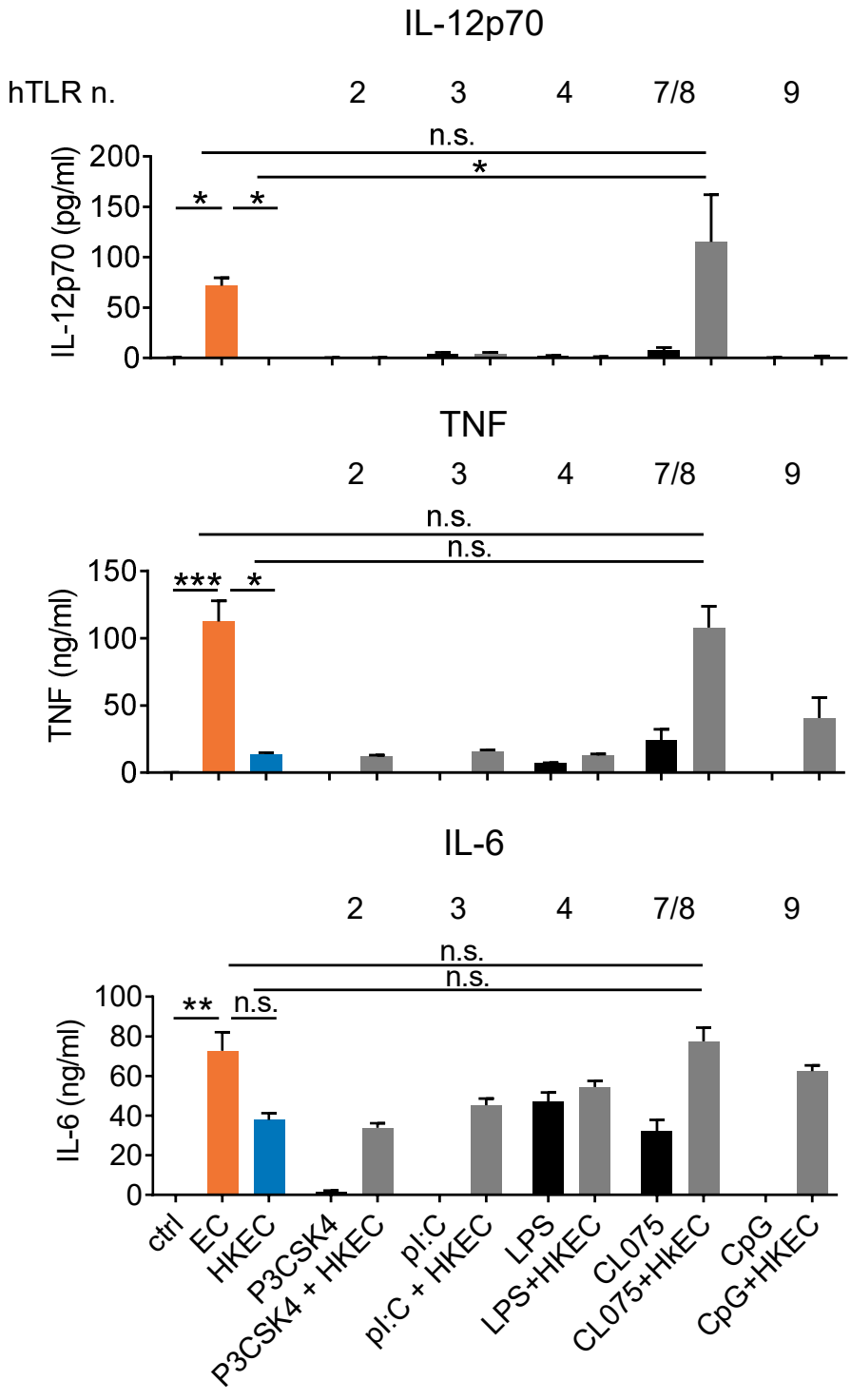


***Figure 14. Involvement of TLRs in sensing microbial viability.*** IL-12p70, TNF and IL-6 measured in supernatants of monocytes (n = 3 experiments with one donor each) either left untreated (ctrl), exposed to EC or HKEC, or the TLR2 ligand Pam3CSK4 (P3CSK4), the TLR3 ligand poly(I:C) (pI:C), the TLR7 and TLR8 ligand CL075 or the TLR9 ligand CpG DNA. Ligands were either used alone or supplemented with HKEC as indicated below the graphs. Bars represent mean + SEM. ⋆ $p \leq 0.05$, ⋆⋆ $p \leq 0.01$, ⋆⋆⋆ $p \leq 0.001$; n.s.=non-significant (one-way ANOVA with post-hoc correction for multiple comparisons).

comparable to viable bacteria when combined with the same infection dose of heat-killed *E. coli* (***Fig. 14***). Conversely, all ligands tested in combination with HKEC induced comparable levels of IL-6. These results clearly pointed towards a potential engagement of TLR7 and/or TLR8 by living bacteria.

As described in the introduction, both TLR7 and TLR8 are located in the early/late endosome and recognize ssRNA and nucleotides combinations derived from RNA degradation[54, 210]. Stimulation of endosomal TLRs such as TLR7 and TLR8 by particulate cargo requires its phagocytosis and endolysosomal degradation. Hence, we used molecular inhibitors of these cellular processes to assess their requirement for the detection of viable bacteria. Cytochalasin D is a fungal toxin, which disrupts actin filament polymerization consequently

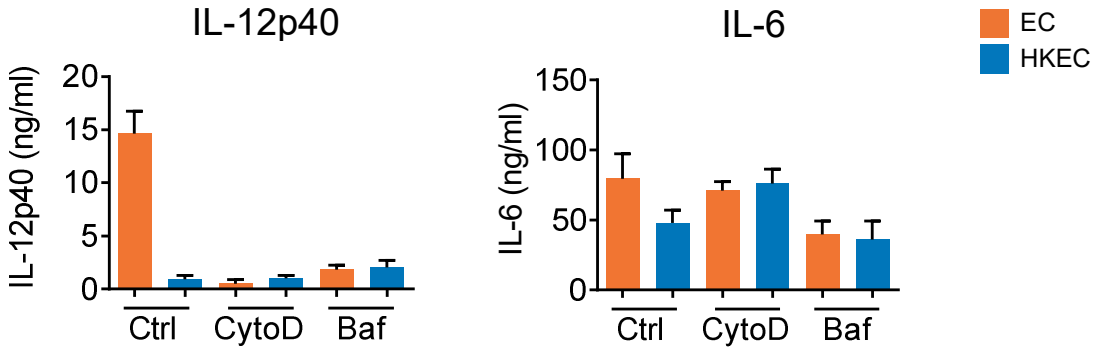


***Figure 15. Phagocytosis and endosomal acidification are necessary for successful detection of viability.*** Human monocytes were left untreated (ctrl) or pre-treated with cytochalasin-D (CytoD) or with bafilomycin A (Baf). Cells were afterward stimulated with EC or HKEC. IL-6 and IL-12p40 release was measured by ELISA (n=3 donors). Bars represent mean + SEM.

inhibits phagocytosis. Bafilomycin A, on the other side, is a specific inhibitor of vacuolar-type $H^+$-ATPases abrogating the progression of endosomal acidification. Both inhibitors abolished EC-induced production of IL-12 but had little or no effect on IL-6 secretion (***Fig. 15***). This observation suggests the importance of endosomal receptors, potentially TLR7 and/or TLR8, in the sensing of viable bacteria. Their endosomal localization positions them to sense bacterial ligands produced during phagosomal degradation of invading microorganisms. IL-6 on the other hand is produced regardless of bacterial viability and through a process that does not involve internalization or degradation of the bacteria, likely via cell surface receptors, e.g. TLR4.

## 3.7 Human APCs detect bacterial viability through bacterial RNA

We reasoned that TLR8 might be preferentially involved in 'viability recognition' and that it might be the primary human *vita*-PAMP receptor for live bacteria since human monocytes

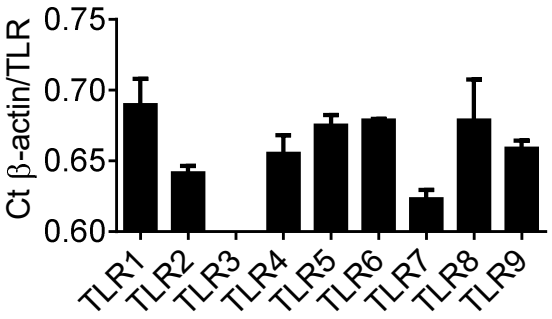


***Figure 16. TLRs expression in human classical monocytes.*** Quantification of the expression of genes encoding for TLR1-9 was measured by RT-qPCR in purified human classical monocytes. Each value is expressed semi-quantitatively as Ct-ratio of house-keeping gene *ACTB* (β-actin)/ *TLR* (n=2 donors). Bars represent mean + SEM.

express high levels of TLR8 but only low levels of TLR7 (***Fig. 16***). Moreover, TLR8 has been recently shown to act as a receptor for bacterial RNA for different species of bacteria[69, 70].

To test this hypothesis, we used *poly-L-arginine* (pLa) to mediate endosomal delivery and retention of bacterial RNA isolated from log-phase culture of *E. coli*. Endosomal delivery of bacterial RNA restored TNF and IL-12 production to levels comparable to those induced by infection with live bacteria (***Fig. 17***), indicating that the recognition of bacterial RNA in human APCs may enable them to discriminate between live and dead bacteria.

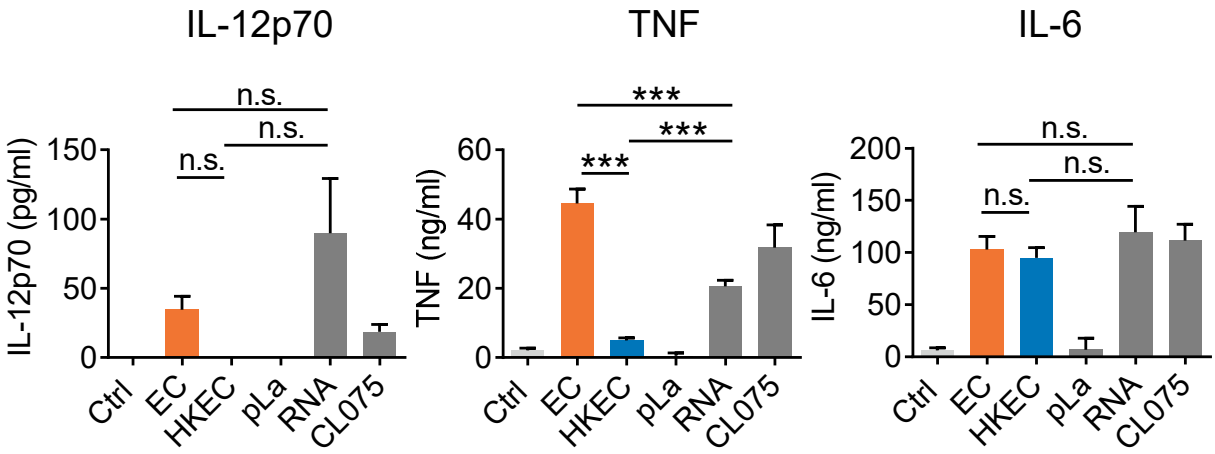


***Figure 17. Human APCs respond to bacterial RNA with a robust production of TNF and IL-12.*** Monocytes were left untreated (ctrl) or stimulated with EC, HKEC, bacterial RNA (RNA) complexed with pLa (polycationic polypeptide poly-l-arginine), CL075 or pLa alone as a control. Cytokines in supernatants were measured by ELISA (n=3-4 individual donors). Bars indicate mean + SEM. ⋆ $p \leq 0.05$, ⋆⋆⋆ $p \leq 0.001$ (one-way ANOVA with post-hoc correction for multiple comparisons).

Besides restoring cytokine production bacterial RNA also induced upregulation of maturation markers (***Fig. 18,*** in collaboration with Jenny Gerhard, Sander group).

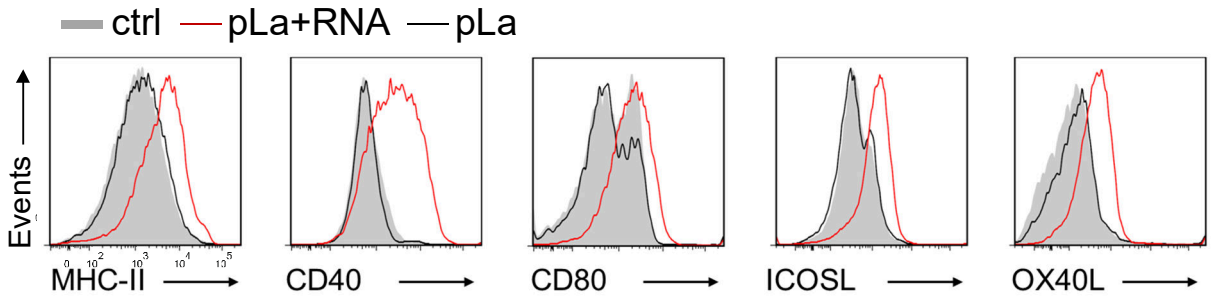


***Figure 18. Detection of bacterial RNA induces expression of monocytes activation markers.*** Human monocytes were left untreated (ctrl), treated with pLa or pLa complexed to purified total bacterial RNA (pLa + RNA). Upregulation of costimulatory molecules and activation markers was measured by flow cytometry 18h post-treatment (n=5 donors)

Supporting the central role of bacterial RNA sensing, we compared several methods of killing known to induce rapid degradation of bacterial RNA content with *paraformaldehyde* (PFA) fixation which, on the contrary, preserves the RNA content[99]. Bacterial killing by heat, UV, or ethanol abrogated the IL-12 inducing capacity, whereas PFA fixed bacteria induced IL-12

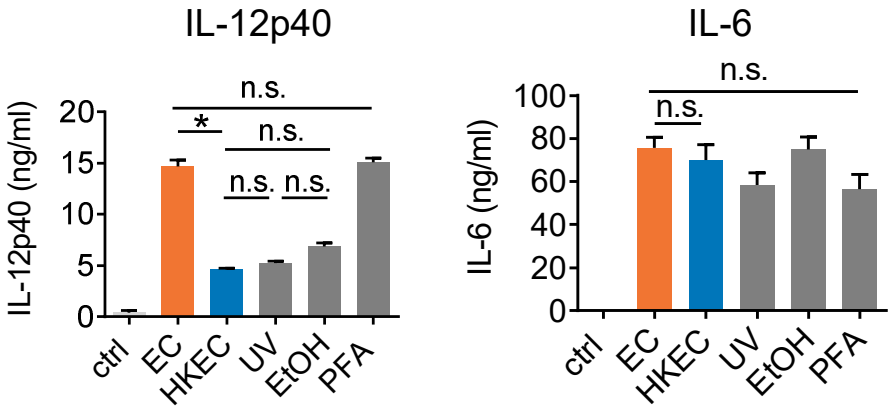


***Figure 19. Effect of different killing methods on the viability-dependent immune response.*** Log phase ThyA$^-$ *E. coli* were inactivated with the indicated method (HK: heat killing at 60°C for 90 minutes, UV: ultra violet irradiation for ~ 20 minutes, EtOH: 70% ethanol treatment for 10 minutes, PFA: paraformaldehyde fixation for 10 minutes) and extensively washed prior to cell treatment. Cytokines were measured by ELISA in the supernatants of APCs left untreated (ctrl) or stimulated with the indicated bacterial preparation at a MOI of 10 (n=3 donors). Bars represent mean + SEM. ⋆ $p \leq 0.05$; n.s.=non-significant (one-way ANOVA with post-hoc correction for multiple comparisons).

secretion similar to that induced by viable bacteria (***Fig. 19***). This indicates that the presence of bacterial RNA is fundamental for an effective viability-dependent innate immune response by human APCs.

## 3.8 TLR8, and MyD88, are necessary for 'viability recognition'

To directly test the involvement of TLR8 during recognition of live bacteria, we silenced the mRNA expression of *TLR8* using *RNA interference* (RNAi).

Primary human monocytes were transfected with three different 20 base-pair long siRNAs

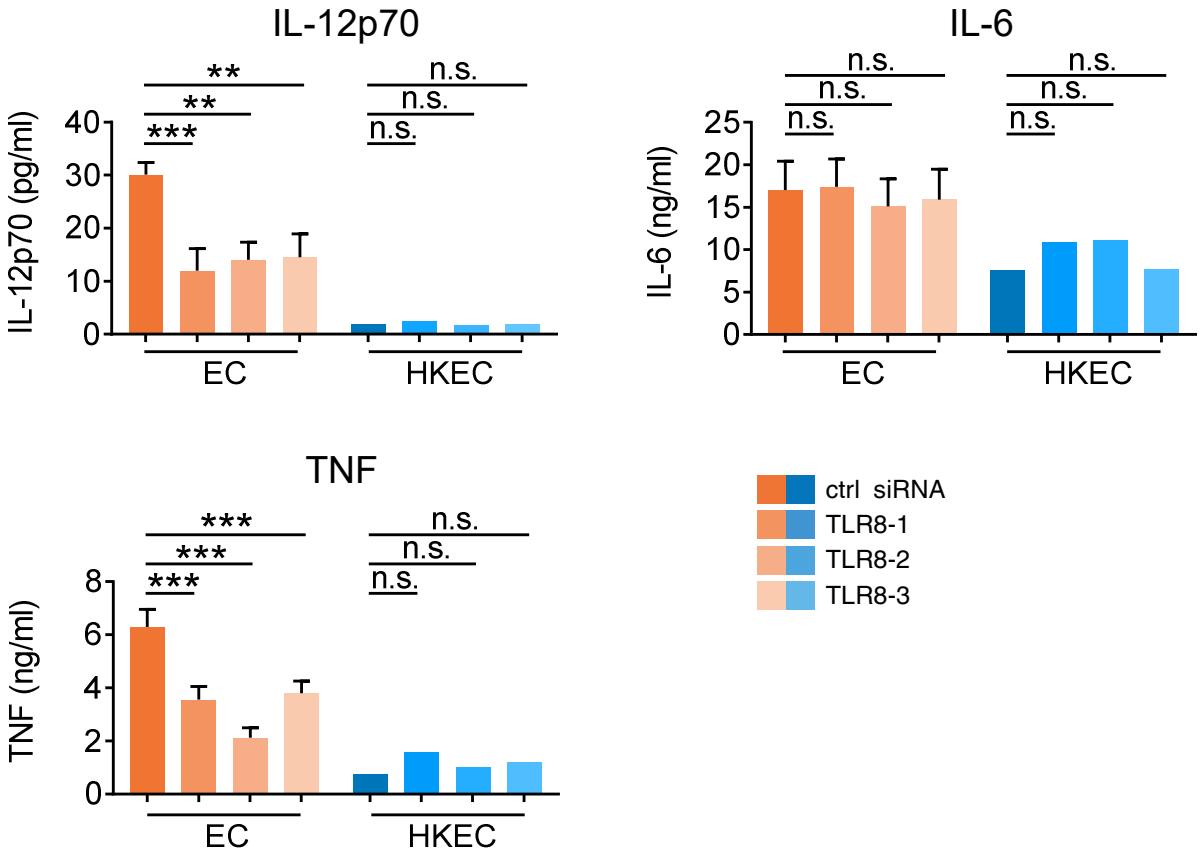


***Figure 20. TLR8 mediates 'viability sensing' in human monocytes***. Cytokines released in supernatants of human monocytes (n=3 donors) pre-treated for 48h either with a control siRNA with a scrambled sequence (ctrl siRNA) or one of three sequence specific siRNAs (1, 2 or 3) directed against TLR8 and subsequently stimulated with EC or HKEC (indicated below plots). Bars represent mean + SEM. ** $p \leq 0.01$ and *** $p \leq 0.001$; n.s.=non-significant (two-way ANOVA with post-hoc correction for multiple comparisons).

directed against TLR8 and its primary signaling adaptor, MyD88. Knockdown of *TLR8* expression diminished the production of IL-12p70 and TNF in response to viable bacteria while had little effect on the secretion of IL-6 (***Fig. 20***), once again supporting the fundamental role of this receptor in sensing viable bacteria. Accordingly, the secretion of TNF, IL12p70 and IL-6 was unaffected by the siRNA treatment when cells were infected with the same infection dose of heat-killed bacteria.

Silencing expression of MyD88, which, as outlined in the introduction, is essential for the signal transduction downstream of TLR8, abolished the production of the proinflammatory cytokines

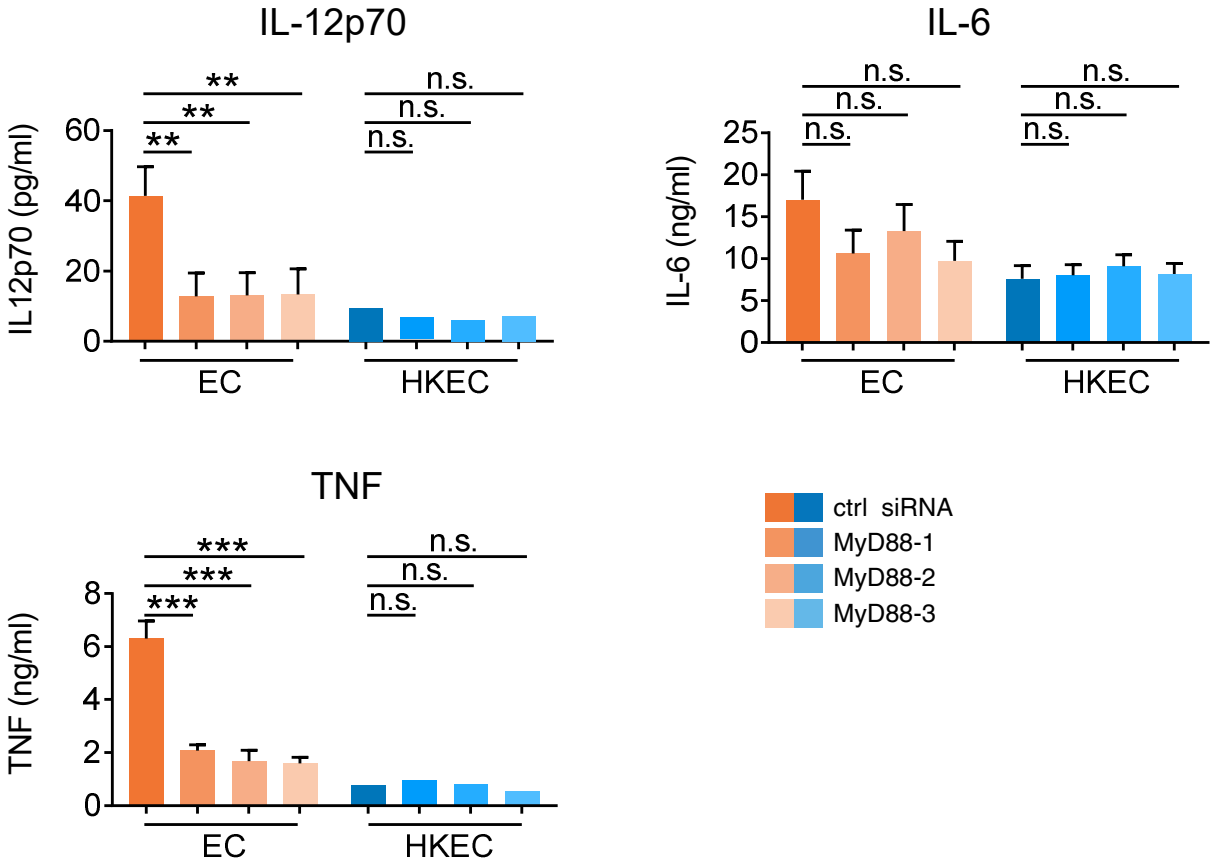


***Figure 21. MyD88 is essential for successful recognition of bacterial viability in human monocytes.*** Cytokines released in supernatants of human monocytes (n=3 donors) pre-treated either with a control siRNA with a scrambled sequence (ctrl siRNA) or one of three sequence specific siRNAs (1, 2 or 3) directed against MyD88 and subsequently stimulated with EC or HKEC (indicated below plots). Bars represent mean + SEM. ** $p \leq 0.01$ and *** $p \leq 0.001$; n.s.=non-significant (two-way ANOVA with post-hoc correction for multiple comparisons).

in response to viable EC (***Fig. 21***). The production of IL-6 was, instead, largely left unaltered by MyD88 silencing (Fig. 21).

Collectively, these results identify TLR8 as the primary sensor for bacterial viability, and strongly support the function of bacterial RNA as a *vita*-PAMP sensed by human APC, leading to the production of IL-12 and TNF.

## 3.9 Detection of bacterial viability promotes $T_{FH}$ differentiation

Based on the obtained results highlighting the role of TLR8 in sensing live bacteria and triggering monocyte-derived cytokine production, we further investigated the impact of *vita*-

PAMP recognition by human APCs on the adaptive arm of the immune system. Given that live attenuated vaccines are known for their notorious superiority over inactivated vaccines, we focused our attention on the signals necessary for the priming of T follicular helper cells. Humoral immunity is largely based on effective interactions between B cells and T helper cells, whereas the role of the innate immune system in this process still remains somewhat understudied. We hypothesized that APCs promote early $T_{FH}$ differentiation by providing the necessary signals and cytokines milieu upon detection of live bacteria.

Development of effector $T_{FH}$ cells, as summarized in the introduction, is a complex and yet not fully characterized process[121, 162] in which various cytokines and cytokine combinations have previously been found to be involved[164-167, 211].

We used a c*onditioned supernatant* system to investigate the role of APC-derived cytokines, induced specifically upon detection of viable bacteria, on the development of $T_{FH}$ cells. To this end, polyclonally activated $CD4^+$ T cells (stimulated with plate bound anti-CD3 and soluble CD28 antibodies) where cultured in the presence of culture media (conditioned supernatant) from APCs previously stimulated with live or heat-killed bacteria. Filtration of the conditioned media prevented carryover of APCs and bacteria, thus allowing the evaluation of secreted factors without cell-cell contact.

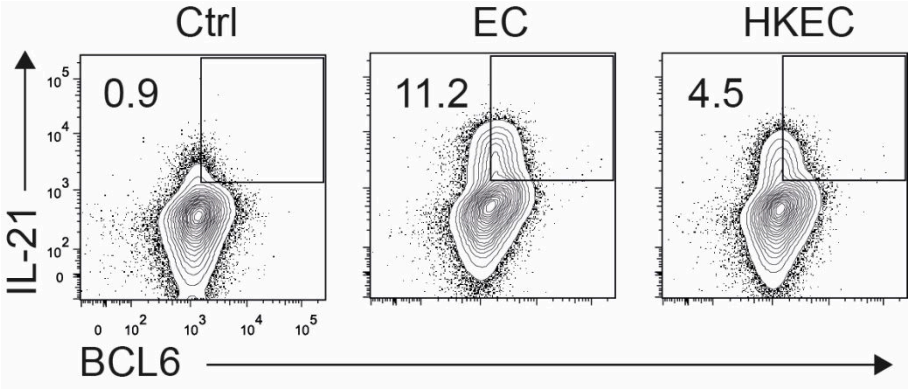


***Figure 22. Viability-induced cytokine production is a critical signal for $T_{FH}$ differentiation.*** $CD4^+$ T cells were polyclonally activated by plate-bound anti-CD3 and soluble anti-CD28 antibodies in the presence of supernatants collected from APCs left untreated (ctrl) or stimulated for 18h with ctrl, EC or HKEC. CD4+ T cells were analysed by flow cytometry for co-expression of bona fide $T_{FH}$ markers BCL6 and Il-21 after 5 days. Data are representative of seven experiments. Numbers reported next to outlined area indicate percent in each throughout.

Culture supernatants from APCs which had been stimulated with live bacteria promoted the expression of IL-21 in T cells, the characteristic cytokine of $T_{FH}$ cells (***Fig. 22,*** carried out together with Jenny Gerhard, Sander group). In addition, T cells also co-expressed high levels of BCL6, the lineage defining transcription factor for $T_{FH}$ cells (Fig. 22). BCL6 expression is necessary for successful $T_{FH}$ cell development[173, 212] and together with the production of IL-21 is

an indicator of lineage commitment. Notably, APCs stimulated with killed bacteria (or control medium) failed to induce IL-21 and BCL6 expression (Fig. 22). The results support a dominant role of APC-derived cytokines, such as IL-12, in the initial stages of $T_{FH}$ differentiation in response to live bacteria.

## 3.10 TLR8 activation in APCs promotes $T_{FH}$ differentiation

Given the pivotal role of TLR8 in the recognition of live bacterial by human APC (Fig. 14 and Fig. 20) we assessed the impact of TLR8 activation in APCs on subsequent $T_{FH}$ cell differentiation. In an experimental setting similar to the one described above, polyclonally activated $T_{FH}$ were stimulated with conditioned media collected from APCs activated with

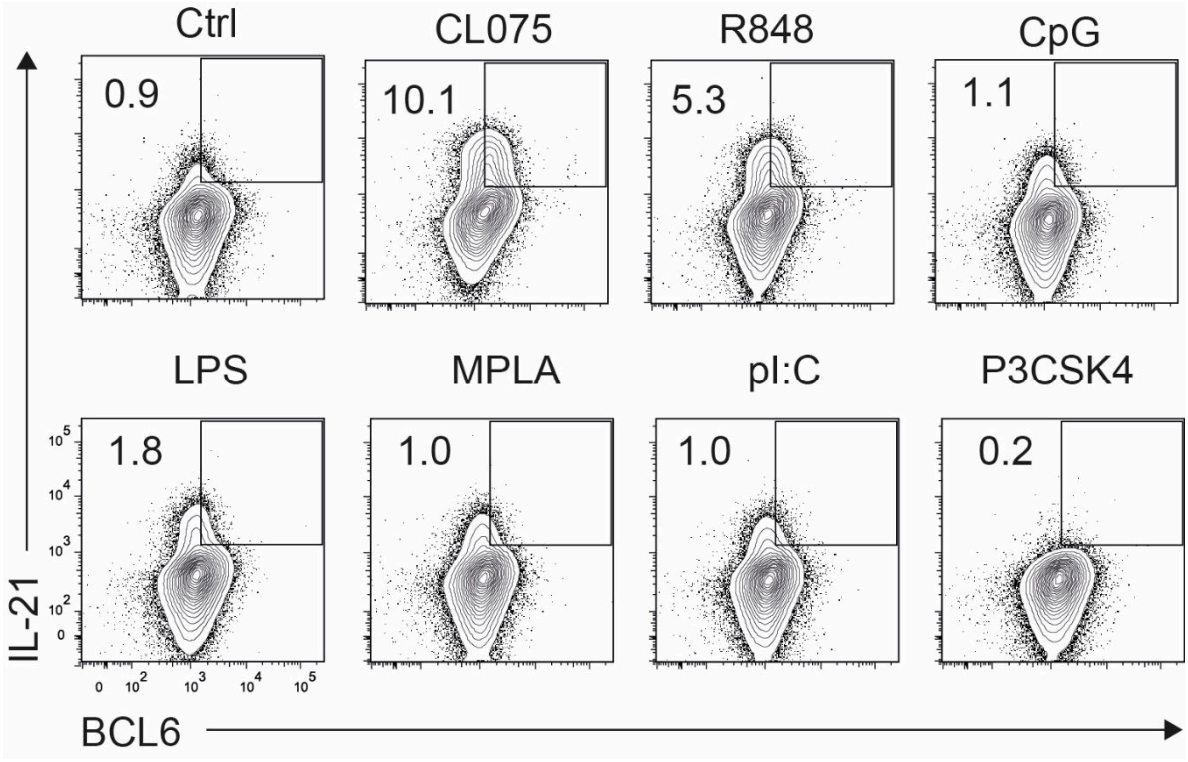


***Figure 23. TLR8 ligation in APCs affects subsequent $T_{FH}$ differentiation.*** $CD4^+$ T cells were stimulated in the presence of culture supernatants from human monocytes previously left untreated (ctrl) or stimulated with the indicated TLR ligands CL075, R848, CpG DNA, LPS, MPLA, poly(I:C) (pI:C) or Pam3CSK4 (P3CSK4). IL-21 and BCL6 expression by $CD4^+$ T cells were detected by flow cytometry after 5 days. Data are representative of seven experiments.

various TLR agonists. Interestingly, only activation of TLR8 (via R848 or CL075) induced $T_{FH}$ cell differentiation (***Fig. 23-24,*** experiments executed together with Jenny Gerhard, Sander group).

Other known TLR ligands were used, including monophosphoryl lipid A (MPLA, aTLR4 agonist) and CpG DNA (a TLR9 agonist), which are currently used as vaccine adjuvants[213], were unable to promote the development of $T_{FH}$ cell phenotype (Fig. 23-24).

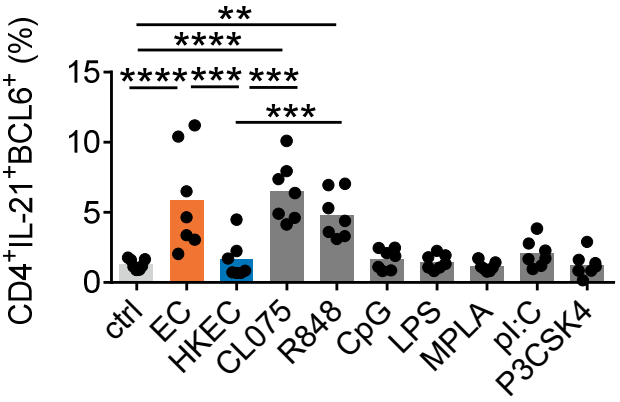


***Figure 24. TLR8 activation by live bacteria in monocytes promotes co-expression of IL-21 and BCL6.*** Quantification of IL-21$^+$BCL6$^+$ cells among T cells cultured with conditioned supernatants derived from APCs treated with culture medium (ctrl) EC, HKEC or the indicated TLR ligands as in Fig. 23. Each symbol represents an individual donor. *, p≤0.05, ** p≤0.01, p≤0.001 and **** p≤0.0001 (one-way ANOVA with post-hoc correction for multiple comparisons). Data are representative of seven experiments.

Our results were also confirmed in a complementary experimental setting taking advantage of a sequential co-culture system in which purified APCs are first stimulated then co-cultured in direct contact with autologous naïve CD4$^+$ T cells in the presence of *Staphylococcal enterotoxin B* (SEB) for five consecutive days. This co-culture system largely reinforced our

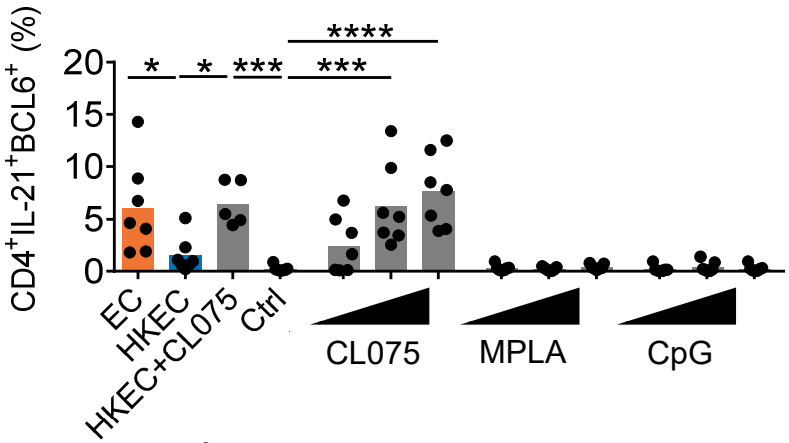


***Figure 25. High concentrations of TLR4 and TLR9 ligands do not induce TFH response.*** APCs were stimulated with control medium (ctrl), EC, HKEC, HKEC supplemented with CL075 or increasing concentrations of CL075 (0.1, 0.5 and 1µg/ml respectively), MPLA (0.1, 0.5 and 1µg/ml respectively), and CpG DNA (0.1, 1 and 2.5µM respectively), and subsequently co-cultured with CD4+ T cells for 5 days in the presence of SEB (T cell antigen receptor stimulus). BCL6/IL-21 expression on T cells was detected by flow cytometry. Each symbol represents an individual donor. * p≤0.05, ** p≤0.01, *** p≤0.001 and **** p≤0.0001 (one-way ANOVA). Data are representative of seven experiments.

conclusions again proving that TLR8 stimulation alone is sufficient to promote the development

of follicular helper cells. Even increasing concentrations of previously used TLR ligands were not sufficient to induce comparable response to the one induced by live bacteria or selective TLR8 activation (***Fig. 25,*** experiments executed together with Jenny Gerhard, Sander group).

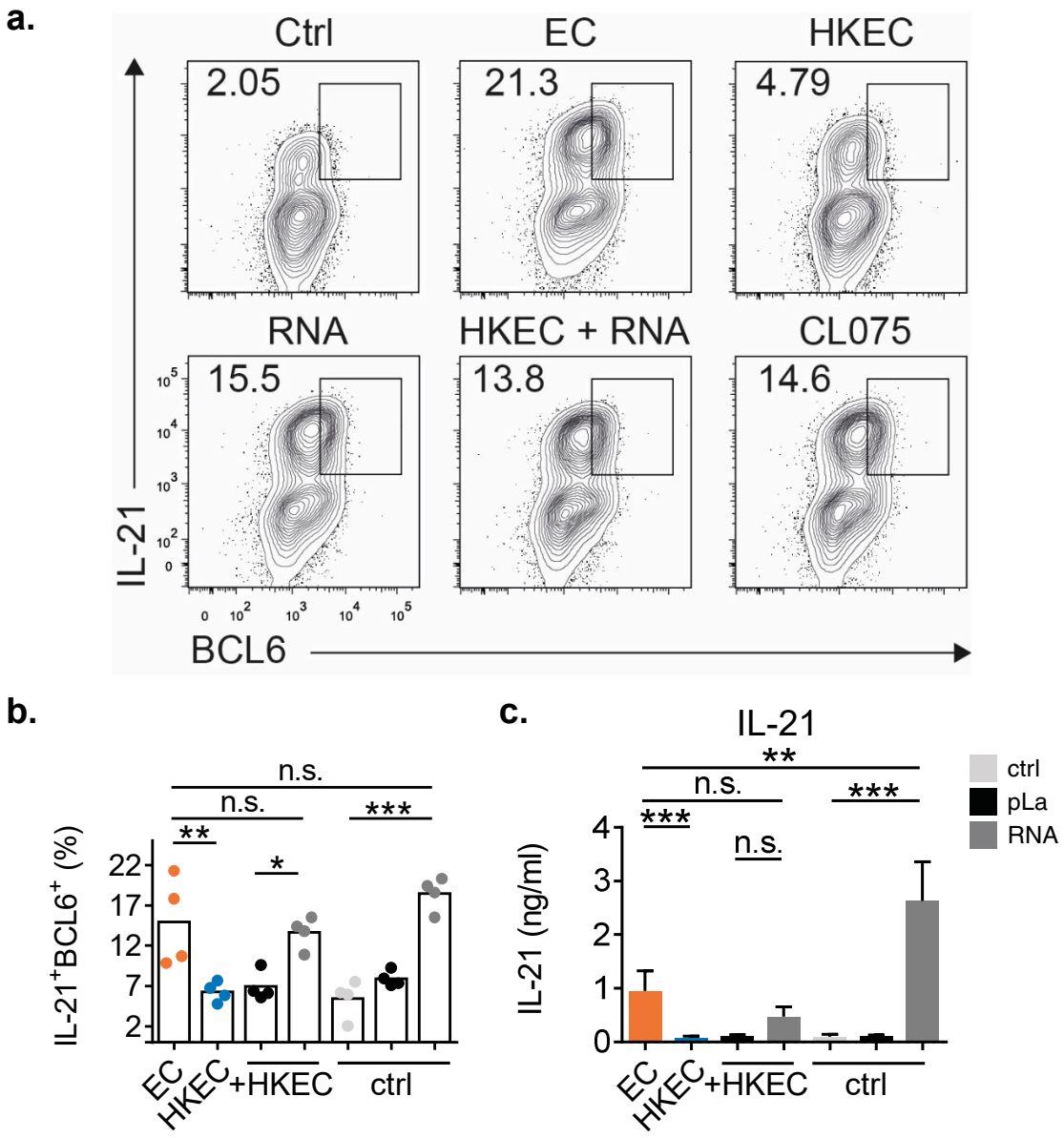


***Figure 26. Sensing of bacterial RNA promotes $T_{FH}$ differentiation.*** APCs were left untreated (ctrl) or stimulated for 18h with EC, HKEC, purified total bacterial RNA complexed with the polycationic polypeptide poly-l-arginine (pLa), HKEC plus bacterial RNA, or CL075 as indicated. Conditioned supernatants were then used to stimulate $CD4^+$ T cells for 5 days. ***(a)*** Flow-cytometry analysis of the expression of IL-21 and BCL6 by $CD4^+$ T cells. ***(b)*** Quantification of the frequency of $CD4^+$IL-$21^+BCL6^+$ T cells after stimulation with conditioned supernatants as in (a) and ***(c)*** IL-21 production in the supernatants of those culture measured by ELISA. Bars are mean + SEM. Each symbol represents an individual donor. * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$; n.s.=non-significant (two-way ANOVA with post-hoc correction for multiple comparisons). Data are representative of four experiments.

## 3.11 Recognition of bacterial RNA induces $T_{FH}$ differentiation

Since the recognition of bacterial RNA was identified as a critical stimulus of APC cytokine responses to living bacteria, we tested the potential $T_{FH}$ stimulatory effect of the culture supernatants of monocytes treated with purified bacterial RNA.

Monocytes were stimulated with total bacterial RNA from *E. coli* or treated with a combination of transfected RNA and simultaneous treatment with HKEC. Activation by purified bacterial RNA indeed led to high levels of $T_{FH}$ cells (IL-21$^+$BCL6$^+$, ***Fig. 26a, b***) and concomitant secretion of IL-21 (***Fig. 26c***, experiments executed together with Jenny Gerhard, Sander group***)***. These results indicate that innate immune recognition of bacterial viability, via TLR8-mediated sensing of bacterial RNA, elicits critical $T_{FH}$ differentiation signals.

## 3.12 TLR8 is required for $T_{FH}$ differentiation in response to viable bacteria

Finally, we investigated the requirement for TLR8 in APCs for their ability to promote $T_{FH}$ commitment. Supernatant of cells previously treated with siRNA against TLR8 (as in Fig. 20), showed a reduce capability to promote $T_{FH}$ differentiation in response to live bacteria (***Fig. 27***,

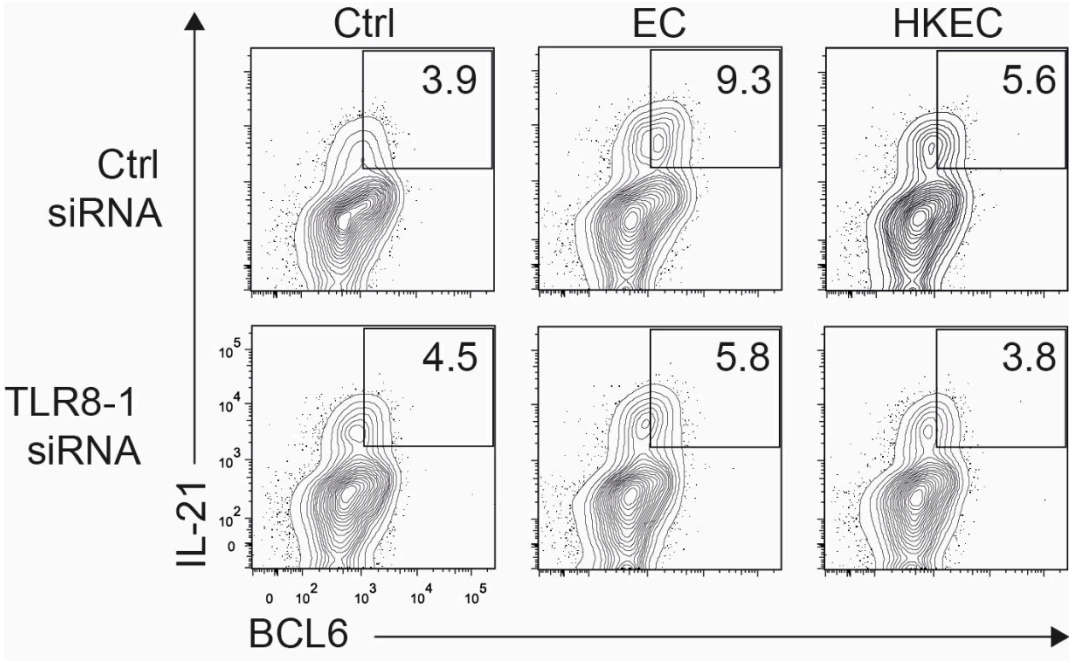


***Figure 27. Silencing of TLR8 reduces APC-mediated $T_{FH}$ differentiation.*** CD4$^+$ T cells were stimulated in the presence of culture supernatants from APCs treated control siRNA (ctrl) or TLR8-specific siRNA (left) and either left unstimulated (Ctrl) or stimulated with EC or HKEC as in Fig. 20. IL-21/BCL6 co-expression by CD4$^+$ T cells was measured by flow cytometry after 5 days of culture. Data are representative of eight experiments.

experiments executed together with Jenny Gerhard, Sander group) when compared to control-treated cells.

Moreover, we observed a reduced secretion of IL-21 in the culture supernatants when $CD4^+$ T cells were stimulated with conditioned media of TLR8-specific siRNA treated APCs (***Fig. 28,*** experiments were carried out together with Jenny Gerhard, Sander group).

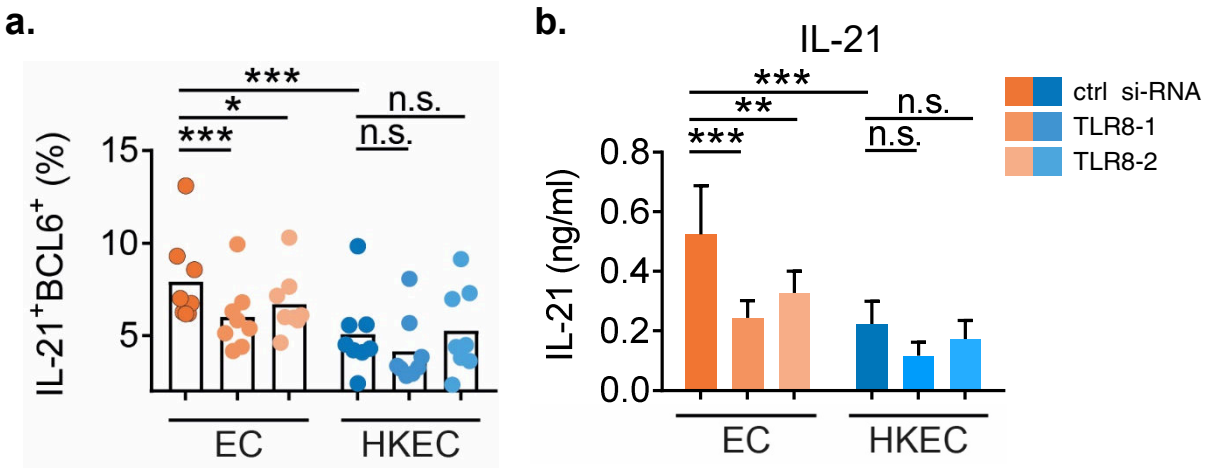


***Figure 28. Impact of TLR8 silencing on APC-mediated $T_{FH}$ differentiation and IL-21 secretion. (a)*** Frequency of IL-$21^+BCL6^+$ T cells in cultures as in Fig. 27 and ***(b)*** ELISA of IL-21 in supernatants of cultures as in Fig. 27. Bars represent mean + SEM. ⋆ $p \leq 0.05$, ⋆⋆⋆ $p \leq 0.001$ (two-way ANOVA with post-hoc correction for multiple comparisons). Data are representative of eight experiments.

In conclusion, TLR8 is a critical sensor for microbial viability and its activation is central for APC-mediated instruction of $T_{FH}$ responses.

## 3.13 Recognition of bacterial viability induces $T_{FH}$ cells in swine

The role of 'viability recognition' has been previously investigated in mice[99], in which detection of *vita*-PAMPs has been shown to alert the innate immune system through activation of the NLRP3 inflammasome, and to enhance humoral immunity. The impact of *vita*-PAMP recognition on T helper cell differentiation has never been examined *in vivo*. To test the applicability of our findings *in vivo*, we turned again to domestic pigs (*Sus scrofa domestica*) due to the significant similarities between the human and porcine physiology and immunity including expression of functional TLR8[214]. Phylogenetic studies using available protein sequences from nine different vertebrate species (including the species discussed in this study; *Sus scrofa*, *Mus musculus*, *Homo sapiens* and *Salmo salar*) showed the close evolutionary relationship between human TLR8 and other mammalian homologues including the porcine and murine ones (***Fig. 29***). The swine TLR8 has a 74% homology with human receptor while

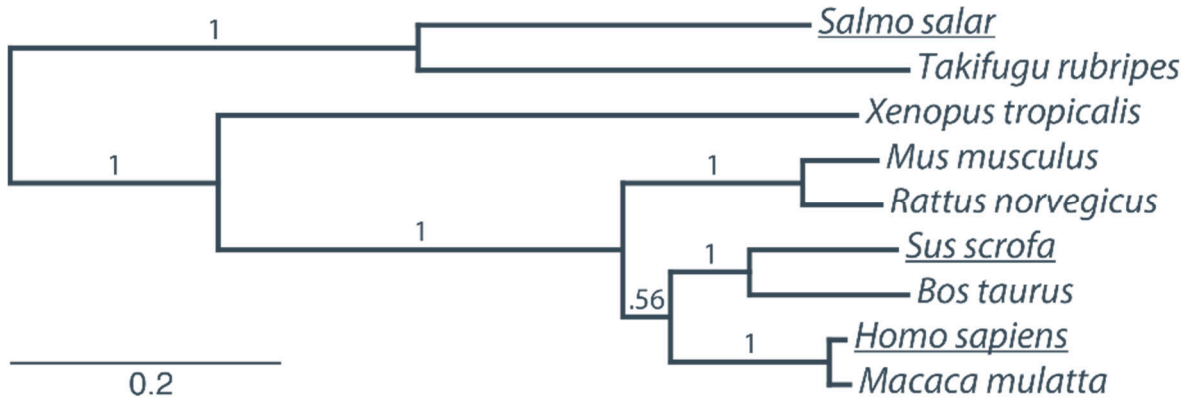


***Figure 29. Phylogenetic tree of TLR8 in nine vertebrate species.*** The sequences were obtained from GenBank (see Materials and Methods). A Maximum likelihood (ML) tree was reconstructed using the phylogeny.fr server using default parameters. Bootstrap values are indicated on the respective branches. The scale represents the substitution rate. Underlined are models examined in this study.

murine TLR8 displays a 66% homology with human and appears to be equally related to the human TLR8 despite the known different recognition capacity of the mouse TLR8.

The rapid emergence of antibiotic resistance in pathogenic bacteria of veterinary relevance has created an urgent need for prophylactic vaccines in pigs in the farming industry. For these reasons, swine are at the same time an appealing animal model and a relevant target population for vaccine studies.

As shown above, porcine monocytes ($CD172^+CD14^+$) and dendritic cells (DC, $CD172^+CD14^-$) discriminate between live and dead bacteria *in vitro* (*E. coli* or *S. typhimurium*, Fig. 11). Similar to human APC, porcine monocytes and DC secreted substantial amounts of IL-12 in response to live bacteria and TLR8 agonist CL075 (Fig. 11). Purified total *E. coli* RNA

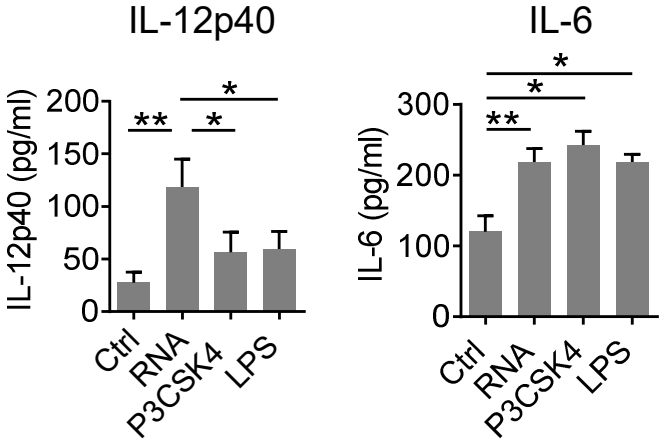


***Figure 30. Porcine APCs are responsive to bacterial RNA.*** Porcine $CD14^+$ monocytes were collected via immunomagnetic isolation from spleens of pigs and stimulated with culture medium (ctrl), bacterial RNA complexed with pLa, Pam3CSK4 (200ng/ml) or LPS (2ug/ml). IL-12p40 and IL-6 production were quantified by ELISA. (n=3-4 experiments). Bars represent mean + SEM. ⋆ $p \leq 0.05$, ⋆⋆ $p \leq 0.01$ (one-way ANOVA with post-hoc correction for multiple comparisons).

also induced the secretion of IL-12p40 in isolated porcine $CD14^+$ monocytes. Interestingly, the same induction was not observed when cells were stimulated with ligands of either TLR2 (Pam3CSK4) and TLR4 (LPS) recapitulating the cytokine secretion profile found in humans (***Fig. 30***).

With the aim of further elucidating whether the process of 'viability recognition' is conserved between human APCs and porcine APCs and, moreover, characterize the involvement of

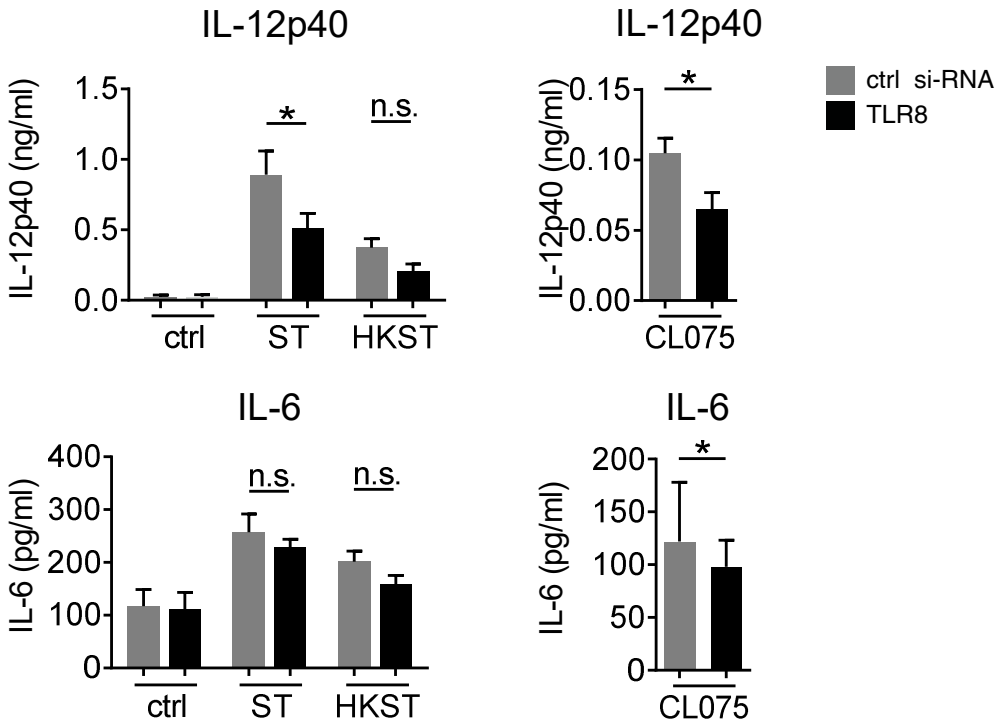


***Figure 31. TLR8 mediates 'viability sensing' in porcine monocytes.*** Porcine splenic $CD14^+$ monocytes (n=3 experiments) were isolated via immunomagnetic isolation and treated with control siRNA with a scrambled sequence or a mixture of four siRNA directed against porcine TLR8 and stimulated after 48h with medium (ctrl), ST, HKEC or CL075. IL-12p40 and IL-6 were measured in the culture supernatants by ELISA 24h post stimulation. Bars indicate mean + SEM. * $p \leq 0.05$; non-significant (two-way ANOVA with post-hoc correction for multiple comparisons).

porcine TLR8, we carried out RNA-mediated interference to abrogate the expression of the gene encoding the swine homolog of human TLR8 in porcine purified $CD14^+$ monocytes. IL-12p40 production in response to live ST was abolished as a result of TLR8 knockdown, as it was the secretion of the cytokine in response to the TLR8 specific ligand CL075. On the contrary, the production of IL-6, whose production is independent from of bacterial viability in the porcine model as well, was unaltered after silencing of the receptor. (***Fig. 31***).

We next investigated porcine $T_{FH}$ differentiation *in vitro*. To this end we deployed a co-culture system not dissimilar to an approach used for human cells[215]. Pig total splenocytes, a heterogenous population including both $CD4^+$ T cells and APCs (such as monocytes and dendritic cells) were stimulated with increasing MOIs of ST or HKST for one hour.

Concanavalin A (to promote polyclonal T cells proliferation) and antibiotics (to halt bacterial replication) were then added to the cultured cells. After four days, we observed a dose-dependent increase in the frequency of $CD4^+IL\text{-}21^+BCL6^+$ $T_{FH}$ cell–like cells when compared to the frequency of the same cells after stimulation with HKST (***Fig. 32a, b***). Similar to human In parallel, we also assessed the ability of soluble PAMPs to promote the development of $T_{FH}$

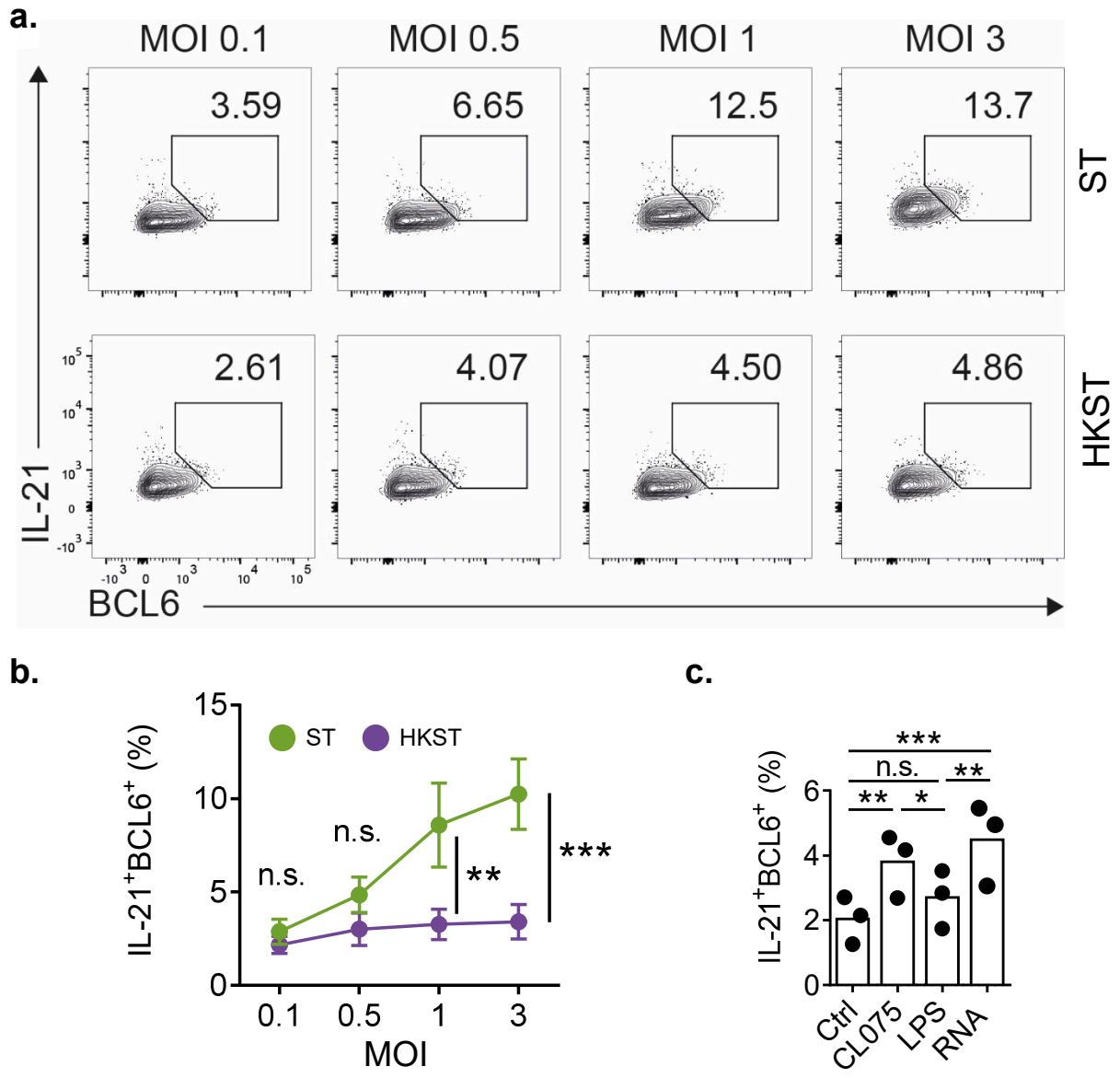


***Figure 32. Porcine $T_{FH}$-like cells differentiation in response to live bacteria stimulation.*** Porcine splenocytes were stimulated for four days with concanavalin A in the presence of ST (top row) or HKST (bottom row) at the indicated increasing doses (MOI). ***(a)*** Expression of IL-21 and BCL6 in $CD4^+$ T cells among the splenocytes was measured by flow cytometry. ***(b)*** Frequency of $IL\text{-}21^+BCL6^+$ cells in cultures described in (a); ST and HKST in green and purple respectively. ***(c)*** Porcine splenocytes either left unstimulated (ctrl) or stimulated with CL075, LPS or bacteria RNA complexed with pLa in the presence of concanavalin A. Frequency of $IL\text{-}21^+BCL6^+$ cells was measured by flow cytometry after 3 days. Error bars are mean ± SEM (b). ⋆ $p \leq 0.05$, ⋆⋆ $p \leq 0.01$, ⋆⋆⋆ $p \leq 0.001$; n.s.=non-significant (two-way ANOVA (b) or one-way ANOVA (c) with post-hoc correction for multiple comparisons). Data are representative of three experiments. FACS analysis in (a) was performed by Sarah Volkers (Sander group).

cells in pig splenocytes cultured *in vitro*. Confirming the role of 'viability sensing' as a pivotal driving force for porcine $T_{FH}$ cell differentiation only the TLR8 agonist bacterial RNA and CL075 induced the expression of $T_{FH}$ cell markers, namely BCL6 and IL-21, in co-cultured autologous $CD4^+$ T cells while TLR4 agonist LPS did not (***Fig. 32c***).

These results replicate the observed phenotype in human cells and they represent the first report of $T_{FH}$-like ($IL\text{-}21^+BCL6^+$) cells in swine.

## 3.14 Bacterial viability drives $T_{FH}$ differentiation *in vivo*

As described in the introduction, $T_{FH}$ cells play a crucial role in the germinal centre reaction leading to production of high affinity antibodies and the generation of immunological memory. We hence set out to investigate the impact of innate immune sensing of 'viability recognition' on $T_{FH}$ cell responses and humoral immunity *in vivo*.

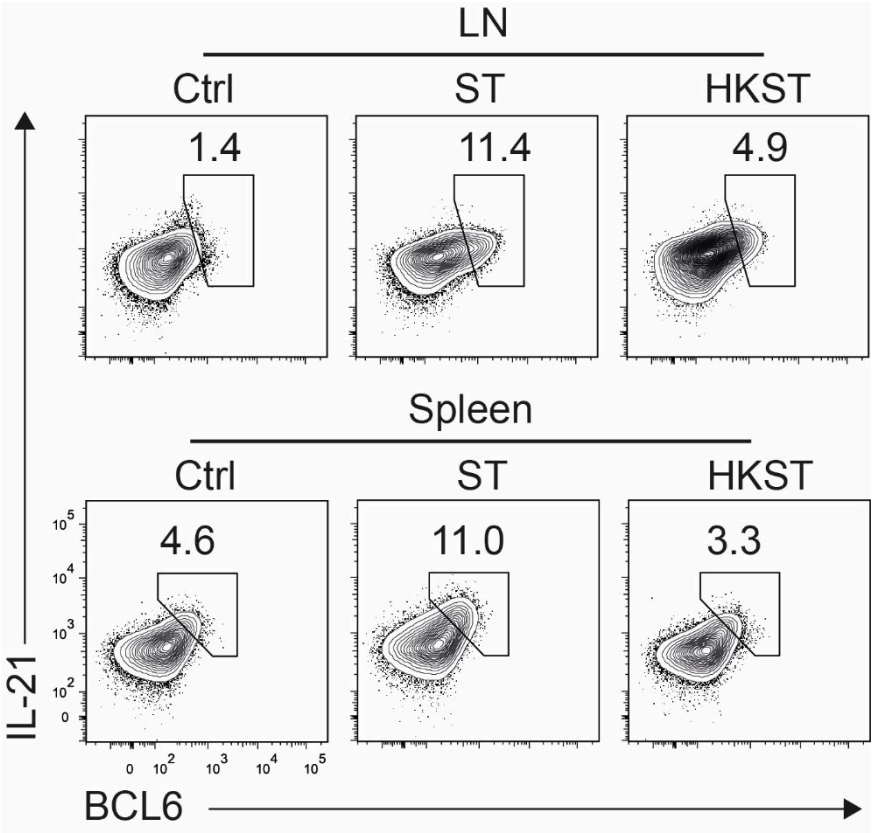


***Figure 33. Porcine $T_{FH}$-like cells arise specifically after immunization with a live Salmonella vaccine.*** Five weeks old pigs were injected subcutaneously with live ST (attenuated Salmoporc-STM vaccine), heat inactivated vaccine (HKST) or saline (Ctrl) and BCL6/IL-21 co-expression in $CD4^+$ T cells was measured by flow cytometry in samples from draining lymph nodes (LN) and spleens on day 30 after immunization. Data are representative of one experiment with five animals per treatment group.

We selected a European Medicines Agency (EMA) approved, commercial live attenuated

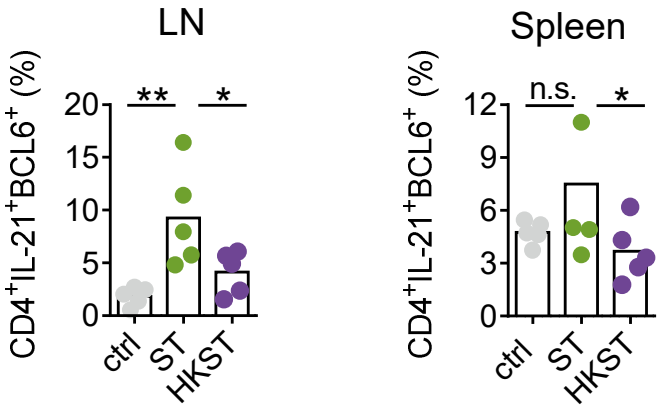


***Figure 34. $T_{FH}$-like cells are increased in spleen and draining LN after vaccination***. Quantification of the frequency of IL-21$^+$BCL6$^+$ cells in lymph nodes or spleens of pigs as in a Fig. 33. Data are representative of one experiment with five animals per treatment group and each symbol represents an individual pig. ⋆ p≤0.05, ⋆⋆ p ≤0.01; n.s.=non-significant (one-way ANOVA with post-hoc correction for multiple comparisons).

vaccine against *Salmonella* infection in pigs. The vaccine, distributed under the trade name of Salmoporc®-STM, contains a live attenuated adenine- and histidine-auxotrophic strain of *Salmonella enterica* serovar Typhimurium (ST) previously used for *in vitro* studies (Fig. 11, 31-32). Five-weeks-old piglets were subcutaneously injected with live Salmoporc®-STM vaccine, with an equivalent dose of heat inactivated vaccine, or solvent as control. Animals immunized with the live vaccine showed an increase in the frequencies of CD4$^+$IL-21$^+$BCL6$^+$ $T_{FH}$-like cells isolated from both the draining (dorsal superficial cervical) lymph node (LN) and the spleen when compared to the animals which received heat-killed bacteria or saline control (***Fig. 33-34***, flow cytometry analysis were performed by Jenny Gerhard, Sander group).

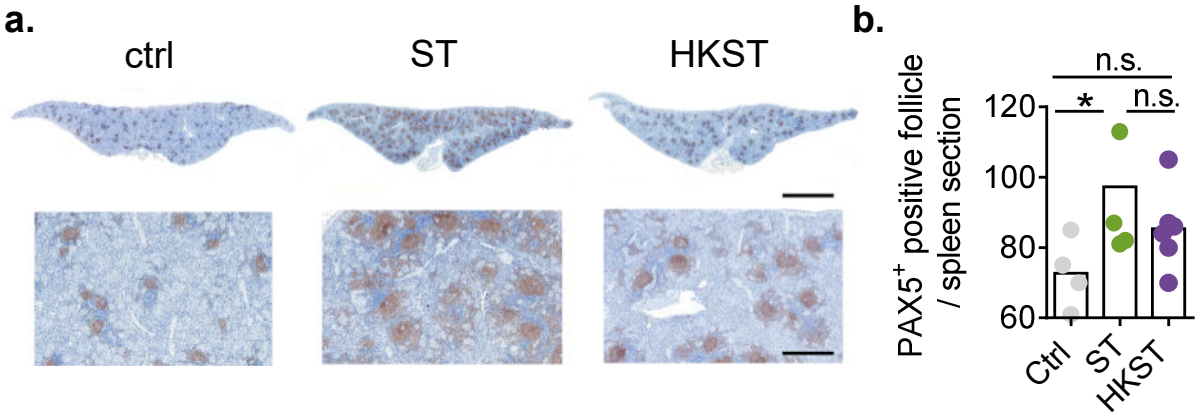


***Figure 35. Increase in the number of PAX5$^+$ follicles after vaccination with a live attenuated vaccine. (a)*** Sections of paraffin embedded spleen tissues were stained for PAX5 to visualize primary follicles after vaccination as in Fig. 33. Scale bars, 5mm (upper panels) or 500µm (lower panels) ***(b)*** Morphometric quantification of PAX5$^+$ follicles in spleen sections of pigs as in (a). ⋆ p≤0.05 (one-way ANOVA). Each symbol represents an individual pig. Histological analyses were performed by Kristina Dietert (Frei Universität, Berlin).

Similarly, the number of PAX5$^{+}$ B cell follicles increased in the spleen of ST vaccinated pigs when compared to the control animals. On the other hand, no significant increase was observed in HKST vaccinated animals compared to the saline controls (***Fig. 35a, b***). PAX5 is a B cell lineage specific protein expressed during development of B cells and by mature B lymphocyte and is a widely used marker to assess the number, size and shape of primary and secondary follicles.

Moreover, we were able to detect higher levels of *Salmonella* (Salmoporc®)-specific *immunoglobulin G* (IgG) in the serum of animal vaccinated with live ST when compared to vaccination with HKST or buffer control, indicating the preferential induction of enhanced humoral immunity in response to the live attenuated vaccine (***Fig. 36***).

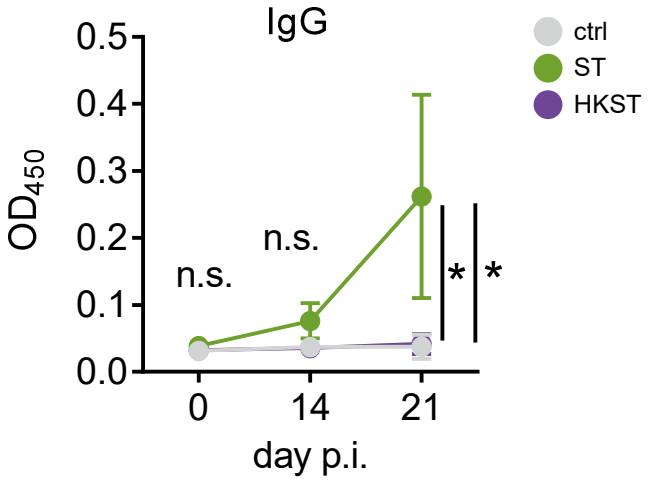


***Figure 36. Salmonella-specific IgG are increased after vaccination with a live attenuated vaccine.*** Anti-*Salmonella* IgG in serum samples of vaccinated pigs as in Fig. 33 were measured by ELISA using *Salmonella* lysates to capture immunoglobulins. IgG levels were assessed before vaccination (day 0) and on day 14 and 21 after vaccination. Data are presented as the optical density at 450 nm (OD450) at a fixed serum dilution. Error bars are mean ± SEM. * $p \leq 0.05$; n.s.=non-significant (two-way ANOVA with post-hoc correction for multiple comparisons). Data are representative of one experiment with three animals per treatment group.

These results strongly corroborated our previous findings with human cells (Fig. 21-26 and Ugolini et al., 2018) and they demonstrated the importance of 'viability sensing', thought recognition of bacterial RNA via TLR, as a driver for $T_{FH}$ cell and follicular B cell responses *in vivo*.

# 4 DISCUSSION

This study provides conclusive evidence to support a central role of 'viability recognition' as a conserved checkpoint regulating both innate and adaptive immunity against bacterial infections. In humans and in swine, the discrimination of living from dead microbes via TLR8 leads to a transcriptional remodelling in APCs and the subsequent selective secretion of cytokines, including IL-12, that strongly promote $T_{FH}$ cell differentiation. As such, targeted activation of $T_{FH}$-polarizing innate immune pathways by specific TLR8-activating adjuvants represents a promising approach to improve vaccine efficacy, given the broad protection afforded by T cell-dependent humoral immunity.

The notorious superiority of live vaccines, or survived infections, at inducing protective immunity when compared to inanimate vaccine preparations has been known for a long time[216]. However, previous dogmas of *self versus non-self*-discrimination as the sole driving force of antimicrobial immunity cannot sufficiently explain the differential response to live and killed microbes, both of which represent 'non-self'[3]. It has been proposed that the immune system discriminates pathogens from less virulent microbes on the basis of conserved pathogenic strategies of infection and invasion called "patterns of pathogenesis"[217]. One of such patterns was postulated to be microbial growth itself with the immune system qualitatively detecting PAMPs signifying either bacterial life or death. However, it was only recently that it was recognized that the innate immune system has, indeed, the inherent capacity to discriminate viable from dead microorganisms, which allows for a more sophisticated fine tuning of the ensuing immune responses[99]. Nonetheless, the role of 'viability sensing' and its underlying molecular mechanisms in the human immune system have remained unknown. It has also been unclear how innate immune recognition of *vita*-PAMPs shapes adaptive immune responses, which ultimately lead to an effective clearance of the invading pathogen and to the establishment of immunological

memory. The present study sheds new light on the molecular mechanisms of bacterial 'viability sensing' and provides important insights into the instruction of adaptive immunity by the recognition of microbial viability.

We showed that this fundamental immunological-checkpoint is conserved across several species. In humans, as well as in swine and in fish, sensing of viable bacteria induces a *pattern* of cytokine production which killed bacteria are unable to promote. We demonstrated how the bacterial RNA receptor TLR8 is crucial in the detection of bacterial viability and, thus, can be identified as the first *vita*-PAMP receptor in humans. Human APC, once activated via stimulation of TLR8, are licensed to become effective stimulators of $T_{FH}$ cell differentiation via upregulation of co-stimulatory molecules and the secretion of $T_{FH}$ skewing cytokines.
Live bacteria, but not inactivated ones, impact the orchestration of a successful $T_{FH}$ cell response, *in vitro* and *in vivo*, thus providing insights on the potential mechanism behind the potency of live attenuated vaccines.

## 4.1 Evolutionary conservation of microbial 'viability recognition'

The process of bacterial 'viability recognition' has previously been dissected in detail only in *Drosophila* and in mice. Fruit flies have been shown to efficiently discriminate live Gram-negative bacteria from dead Gram-negative[209]. A *regulatory peptidoglycan recognition protein* (rPGRP-LC) was shown to be responsible for dampening the immune response following recognition of polymeric peptidoglycan, a hallmark of cell wall degradation and thus bacterial lysis. Monomeric peptidoglycan, on the other side, as a byproduct of active bacterial growth, signals active bacterial replication and thus elicits a strong immune response activating the *immune deficiency* (IMD) pathway promoting inflammation and secretion of antibacterial peptides. The regulatory protein is responsible for a timely resolution of the immune response, once the infection is properly cleared, limiting the potentially lethal effect of an immune over-stimulation[209]. In mice, on the other hand, bone marrow derived macrophages actively sense live bacteria over dead bacteria by recognition of *vita*-PAMPs. Thus, instead of exploiting ligands specific to dead microbes to terminate immune reactions after pathogen clearance[99], murine APCs recognize bacterial mRNA as a signature of microbial life, and they actively respond by secreting high levels of IL-1β and IFN-β in a TRIF dependent manner[99]. A recent report, which was published in parallel to our study[215], showed that murine APCs sense bacterial RNA from Gram-negative bacteria in a TRIF dependent manner leading to a hierarchical

secretion of IFN-β and IL-1β, which, in turn, drives the commitment of naïve $CD4^+$ T cells to the $T_{FH}$ fate[218].

While the checkpoint of 'viability sensing' itself seems to be largely conserved between mice and humans, there are important differences regarding the molecular intermediaries and responses elicited by bacterial viability. Our study uncovers an additional layer of regulation of human immune responses by *vita*-PAMP recognition. Whereas in mice 'viability recognition' mainly results in post-transcriptional regulation including NLRP3 inflammasome activation and increased production of IL-1β in response to live bacteria[99, 218], human APCs are regulated on both transcriptional and post-transcriptional levels. Similar to murine APC, live bacteria induce IL-1β production in human APCs as well, but additionally recognition of live bacterial and bacterial RNA results in a selective transcriptional remodeling and the production of inflammatory cytokines IL-12 and TNF, both of which are induced regardless of bacterial viability in mice (Fig. 4-5). At this point it is unclear whether inflammasome activation is induced by direct sensing of bacterial RNA in the cytosol or by indirect activation via endosomal sensing of RNA[219].

The usage of TLR8 in human and porcine APCs represents a critical difference to *vita*-PAMP sensing in murine cells. Murine TLR8 is irresponsive to ssRNA[64] and to the commonly used synthetic TLR8 agonists (imidazoquinolines[50]). However, it was shown to be activated by a combination of imidazoquinoline CL075 (also called 3M-002) and with poly(dT) oligonucleotides (ODNs), which mediated NF-κB activation and secretion of TNF[51]. Recently it has been also proposed that TLR8 can be activated in murine pDCs by vaccinia virus DNA[220]. However this finding has raised several controversies since TLR8 has s not been previously linked to DNA detection, and, moreover, murine and human pDCs do not generally express TLR8[52]. These discrepant functionalities of human and murine TLR8[221] might explain some of the distinct mechanisms involved in microbial 'viability recognition' in humans and mice. Additionally, mice express TLR13, a recently characterized receptor for conserved bacterial ribosomal 23s RNA[222, 223]. This receptor is not present in humans, and while TLR13-dependent recognition was involved in stimulation by purified RNA, it was redundant if live bacteria were used as a stimulus[224]. Different phagocytic properties or differences in the nucleic acid internalization or the availability of yet unidentified RNA surface receptors (which are known for both dsRNA[225, 226] and ssRNA[227]), could also provide an additional level of regulation in the two species and require additional investigation in the context of microbial infection. Hence, the exact process of bacterial 'viability sensing' in mice and the contribution of the endosomal receptors TLR7, TLR8 and TLR13 still remains to be fully resolved.

While in mice only bacterial mRNA, but not tRNA or rRNA, is able to activate the NLRP3 inflammasome, human APCs respond to all the three tested subclasses of bacterial RNA[101]. It is currently unknown if this promiscuity of ligands holds true also for the TLR8-mediated recognition of bacterial RNA in human cells, also in light of the reported sequence specificity of this receptor[58]. The recent structural characterization of the TLR8 ligand capability at the structural level opens to interesting hypothesis over its role in the sensing of live bacteria[54, 215]. TLR8 has been shown not to recognize intact ssRNA, as previously thought, but instead its degradation products in the form of a uridine mononucleoside and a short oligonucleotide (longer than a 2-mer and containing a purine base) at two distinct binding sites on the receptor surface[54]. This finding is in accordance with the role of the 'viability sensor' we propose, since, due to its endosomal localization, it can effectively detect degradation products of lysosomal

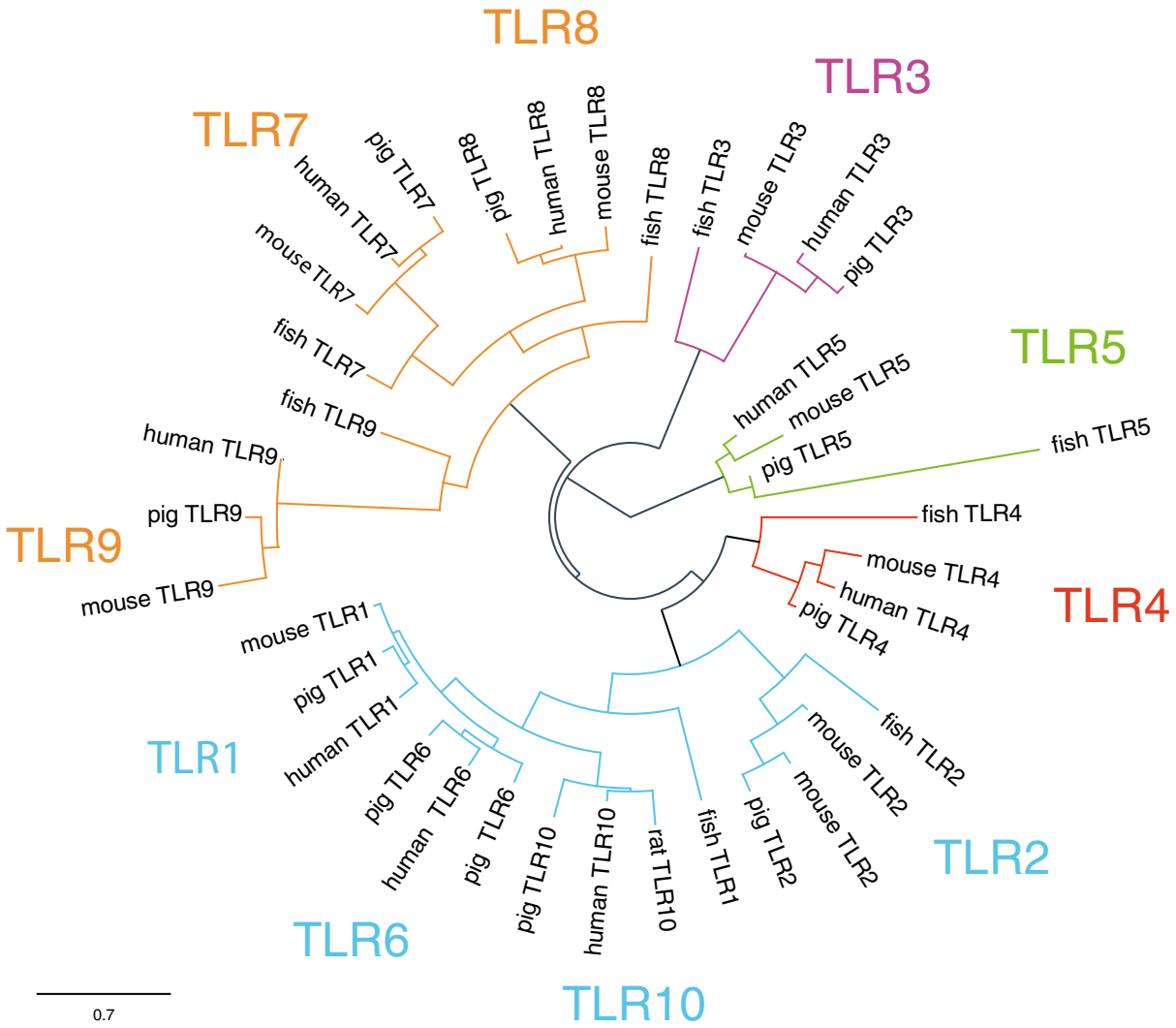


***Figure 37. Phylogenetic tree of human, murine, porcine and piscine TLR.*** The maximum likelihood tree was constructed using annotated sequence of indicated TLRs from *Homo sapiens*, *Mus musculus*, *Sus scrofa domestica* and *Salmo salar*. When homologue of the human TLR was not present, or only predicted in the salmon lineage its sequence was replace by other teleost representatives: TLR2 from *Oncorhynchus mykiss* (Rainbow trout) and TLR4 from *Danio rerio* (Zebrafish). The scale represents the substitution rate.

nucleases and phosphatases. The nature and activity of novel ribonucleases/phosphatases involved in this degradation of ssRNA or the involvement of know cellular hydrolytic enzymes (such as RNase-L[228] or RNaseT2[229]) remains to be elucidated.

We were able to show the evolutionary conservation of 'viability sensing' also in the Atlantic salmon *(Salmo salar)* and the swine *(Sus scrofa domestica)*, two evolutionarily distant species. Vertebrate TLR families share different degrees of sequence similarities but cluster together when assembled into a phylogenetic three (***Fig. 37***). The phylogenetic tree shows five major families encompassing nearly all vertebrate TLRs (six families if including mice TLR11, TLR12, TLR10 and teleost fish TLR13, 19, 20, 21, 22 and 23 which have been excluded here, since the group is represented in humans only by a pseudogene[23]). The phylogeny of each major TLR family (including the TLR8 subgroup represented in Fig. 29) essentially mirrors the phylogeny of the respective vertebrate species and reveals a high degree of similarity and a slow evolution[230], probably as a consequence of a strong selective pressure in safeguarding the recognition of PAMPs. The four salmon orthologues of TLR8 (TLR8a1/2 and TLR8b1/2)[231] have been shown to be highly expressed in immune relevant tissues (spleen and head kidney)[232], and are differentially modulated in primary head kidney cells (and derived cell lines) by cytokine and interferon treatment[231, 233]. Although additional studies are certainly needed to identify the ligand specificity of the salmonid TLR8, it is noteworthy that leukocytes in the closely related rainbow trout do respond to R848 [234], inducing cytokine production and that the receptors expression patterns change after infection with *infectious salmon anemia virus* (ISAV; a ssRNA virus)[232].
Interestingly, we could show that the discrimination of bacterial viability holds true even in the distant teleost fish lineage. Salmon, like other fish species, possess equivalents of mammalian immune cells (B/T lymphocytes, monocyte macrophages, dendritic-like cells and granulocytes[235]). Monocytic cells isolated from peripheral blood circulation (Fig. 12), indeed, respond selectively to live *E. coli* by inducing IL-12 (two of the three identified paralogues), TNF-α and IL-1β, while IL-6 expression is induced in response to both stimuli (Fig. 13), similar to responses in human monocytes.
In swine, as expected, given the already mentioned broad similarity to humans, 'viability sensing' is also conserved. Live bacteria (*E. coli* or *S. typhimurium)* drive the production of IL-12, while IL-6 is produced regardless of viability, both in monocytes and DC (Fig. 11). Moreover, pig monocytes and DCs effectively respond to the human TLR8 ligand CL075 and to bacterial RNA (Fig. 30).

Recently, it was demonstrated that human neutrophils can also detect bacterial viability and activate bactericidal mechanisms (i.e. release of *neutrophils-extracellular traps*, NETs) selectively

upon detection of prokaryotic RNA[236] supporting a role for 'viability sensing' that extends beyond monocytes and dendritic cells to other immune cells. Nonetheless, the involvement of other cell types in mice and swine remains to be investigated.

In conclusion, recognition of microbial viability seems to be an ancestral quality of the innate immune system that is highly conserved, albeit with different modalities from insects, to fish and to mammals. Different species, which occupy different environmental niches and possess immune system with different functionalities bearing different arrays of receptors, share the ability to respond to microbial viability.

## 4.2 The role of vita-PAMPs recognition on $T_{FH}$ cell responses and humoral immunity

While the interactions between T helper cells and B cells leading to the successful generation of antibody responses are well characterized, initial priming events and the $T_{FH}$ skewing properties of innate immune stimuli have been less well understood.

In general, it is well accepted that APCs are crucial in the priming of naïve T cell and initiation of $T_{FH}$ responses[160]. APCs perform this role essentially providing three complementary signals to the T cells after their migration from the periphery to the T cell-rich zone of the secondary lymphoid organs: (i) MHC-II antigen presentation (ii) expression of co-stimulatory molecules (iii) secretion of $T_{FH}$ promoting cytokines. While it has been shown that continuous antigen presentation is necessary for $T_{FH}$ development[160] (provided in our experimental conditions by targeted TCR activation via a superantigen), we focused our attention on signals (ii) and (iii).

The study reveals that live and dead Gram-negative bacteria equally induce the expression of CD40, CD80, OX40L and ICOSL on APCs (Fig. 9), which had to be expected given the abundance of bacterial PAMPs in both preparations. Interestingly, upregulation of the same markers was also prompted by stimulation with purified bacterial RNA (Fig. 18) confirming the immunostimulatory potential of this microbial nucleic acid. Finally, the third signal is provided by the creation of a viability specific cytokine milieu, particularly the release of IL-12, an essential driver of $T_H1$ and early $T_{FH}$ responses[166].

It has been proposed that any given microbial stimulus or PAMP is likely to induce $T_{FH}$ differentiation[121] due to the broad applicability of high affinity antibodies in the defense against most pathogens. This study challenges this wide-spread notion. Our findings show that primary human $CD14^+CD16^-$ monocytes, as well as porcine monocytes and DC, secrete high amounts of IL-12 in response to live bacteria and TLR8 ligation (Fig. 5 and 11). Other TLR ligands or

heat-killed bacteria are poor inducers of IL-12 production (Fig. 14, 30). Interestingly, combination of heat-killed bacteria supplemented with TLR8 agonist CL075 induced IL-12p70 secretion at amount comparable to live bacteria (Fig. 14), further confirming the validity of this TLR8 agonist as immunostimulatory adjuvant for inactivated bacterial vaccines.

TLR agonists have been previously reported to promote $T_{FH}$ differentiation. The expression of OX40L, a surface molecule linked to $T_{FH}$ differentiation[237], was observed in human myeloid APCs after stimulation with RNA-containing immunocomplexes characteristic of systemic Lupus erythematosus (SLE)[170]. Monocyte-derived dendritic cells boost $T_{FH}$ differentiation in mice when stimulated with adjuvants activating TLR9 (i.e. CpG)[238]. Moreover, again in mice, adjuvant use of TLR3 ligands (alone or in synergy with other TLR agonists[239]) results in APC activation[240] and increased humoral antigen-specific immune responses supported by an increase in the frequency of $T_{FH}$ cells[241, 242]. TLR3 activation, in particular, has been shown to mediate the upregulation of costimulatory molecules CD40, CD80 and CD86 (but not ICOSL) on the surface of APCs in a type I interferon depended mechanis[243]. However, mice and human $T_{FH}$ cell differentiation is very distinct with regards to cytokine requirements, thus hampering the translation of the results from the mouse model to humans.

Here we demonstrate that CpG DNA oligonucleotides or poly(I:C) only promote weak $T_{FH}$ development when compared to stimulation with viable bacteria or TLR8 agonists (Fig. 24, 25). In particular, MPLA (TLR4) and CpG (TLR9) failed to induce a $T_{FH}$ commitment even at high concentrations in our system (Fig. 24, 25). *In vivo*, a heat-killed vaccine, which is known to contain large quantities of PAMPs, including LPS and bacterial cell wall components and bacterial DNA, did not promote $T_{FH}$ cell differentiation (Fig. 33), further emphasizing the dependency of $T_{FH}$ cell responses on bacterial viability and *vita*-PAMPs. A study form 2009 showed that whole heat-killed bacteria and bacterial LPS were sufficient to induce $T_{FH}$ cell differentiation by human *in vitro* differentiated Mo-DC[165]. However, the experimental condition used to generate *in vitro* Mo-DC uses media containing high amounts of IL-4, which is known to make DC prone to produce large quantities of bioactive IL-12[244]. In contrast, primary monocytes failed to induce significant $T_{FH}$ responses in response to killed bacteria and LPS (Fig. 24).

As previously mentioned, the cytokine requirements for successful $T_{FH}$ differentiation varies substantially from mice to humans. In rodents IL-6, IL-21 and IL-27 play a central role and loss of these cytokines strongly reduces, but does not completely abolishes, the number of $T_{FH}$ cells[245]. This once more underlines the evolutionary divergence of pathways between mice and humans where the role of IL-6 is marginal[246] and IL-12, IL-23 and TGF-β are the driving

$T_{FH}$ cytokines. In particular IL-12 via activation of STAT4[165, 167], (and marginally of STAT3[177]) seems to play a central role to this process.

Confirming this central role for IL-12, our study shows that silencing of TLR8 in APCs reduces the secretion of IL-12 by human and porcine monocytes in response to live bacteria (Fig. 20 and 31) which translates into a reduction of the $T_{FH}$ stimulating abilities of the silenced APC (Fig. 27).

Further evidence stems from studies in individuals carrying genetic deficiency in *IL-12RB1* (the receptor for both IL-12 and IL-23). These individuals show impaired $T_{FH}$ development[167], fewer memory B cells and antibodies with lower affinity, while overall antibody levels remain largely normal. Other studies have found a less pronounced $T_{FH}$ reduction, but it must be noted that they included much fewer subjects[168, 177]. Therefore, while we demonstrated that IL-12 is necessary and may act in concert with other cytokines, better characterization of the pathways leading to $T_{FH}$ development in patient with primary immunodeficiencies (such as defects in the adaptors MyD88 or IRAK4) are needed.

More recently, an elegant approach based on a high-throughput screening has resulted in the identification of activin A as new potent stimulator of the $T_{FH}$ cells early decelopment[247]. This pleiotropic cytokine belonging to the TGF superfamily[248] was shown to act together with IL-12 and lead in the acquisition of a complete $T_{FH}$ phenotype[247]. Activin A is produced also by monocytes[249], it is greatly enhanced after interaction with cognate T cells in the bone marrow and it is also upregulated by IL-1β stimulation[250]. The secretion of activin A in response to bacterial viability was not evaluated in the current study, yet it is intriguing to speculate that IL-1β, via an autocrine loop, could promote its secretion from classical monocytes further enhancing the $T_{FH}$ differentiation potential.

## 4.3 Large animals as valuable systems for vaccine research

The development and, most importantly, pre-clinical evaluation of vaccine adjuvants, can be problematic due to the sometimes-limited transferability of findings between laboratory animals and humans. Mouse APCs express different PRRs than human APC, and importantly, murine TLR8, as noted, does not recognize the same agonists as human TLR8[51]. For this reason, several studies resorted to non-human primates and humanized mice as model organisms to study the effects of TL8 activation *in vivo*. Here we show that the swine immune system is responsive both to bacterial viability and TLR8 ligands (Fig. 11, 30) and could, thus, represent a valid pre-clinical model for vaccine testing. In addition, there is a growing need for efficacious (non-

rodent) veterinary vaccines, which makes the pig an attractive species for vaccine research. It has been shown that TLR8 targeted adjuvants conferred higher protection against *Porcine reproductive and respiratory syndrome virus* (PRRSV), but the underlying mechanisms at the cellular lever have not been investigated[251]. These strong advantages of the porcine system could outweigh some of the obvious challenges of large animal vaccination studies, such as limitations in group size or scarcity of advanced experimental tools, namely transgenic breeds, when compared to mice. Additionally, Non-specific-pathogen-free (SPF)-housing and outbreeding of the swine increases the significance of a meaningful comparison to humans.

We have demonstrated in a porcine vaccination study, that a well-established live attenuated *Salmonella* vaccine, but not its killed counterpart, constitutes a potent inducer of $T_{FH}$ responses (Fig. 33-34) It also induces stronger B cell follicle formation in secondary lymphoid organs (Fig. 35) and antibody production when compared to the control animals or animals receiving an equal dose of heat-killed bacteria (Fig. 36). Our study represents, to our knowledge, the first evidence of $T_{FH}$-like ($BCL6^{+}IL\text{-}21^{+}$) cells in the swine and dissects their induction upon recognition of bacterial viability both *in vivo* and *ex vivo.* In the latter, in a co-culture setting, we show the ability of splenocytes stimulated with live bacteria to induce the differentiation of $T_{FH}$ cells (Fig. 32). Currently we are also developing a follow-up study in which inanimate vaccine preparations will be combined with a specific TLR8 agonist or bacterial RNA to directly evaluate their potency as vaccine adjuvants and their effect in in the outbred pig model.

Further corroborating the link between the detection of bacterial viability via TLR8 and vaccine efficacy, we investigated the role of a hypermorphic TLR8 single nucleotide polymorphism (SNP; rs3764880[252]) in vaccine responses to a live bacterial vaccine. TLR8 has previously been shown to be up-regulated after phagocytosis of *M. bovis* by THP-1 cells[253]. In a collaboration with the Department of Microbiology of the Charité Universitätmedizin Berlin (Prof. Dr. Ralf R. Schumann and Ms. Sanne Burckert) we discovered a link between the aforementioned functional TLR8 SNP and increased protection associated with BCG vaccination in early life[215]. The SNP alters the starting ATG codon into a GTG triplet[252] shifting reading frame and forcing a second in frame ATG (M4) to be used as alternative start codon. Model simulation showed how this polymorphism might affect both the signal peptide (shortened by three amino acids) and the ligand capability of the receptor altering the accessibility of functional pockets and clefts on the binding surface. The impact on receptor activity could potentially explain the variable outcomes of BCG-vaccination reported in several studies[254].

## 4.4 Impact for vaccine and adjuvant design

The present study highlights a central role of TLR8 in the process of 'viability recognition'. Induction of IL-12 and TNF are similarly driven by the recognition of by live bacteria and RNA, which we identified as a potent *vita*-PAMP in humans and swine. Silencing of TLR8 expression impairs the recognition of bacterial viability in human (Fig. 20) as well as in porcine APCs (Fig. 31). The combined cytokine milieu and co-stimulatory landscape following recognition of bacterial viability in APCs, in turn promote $T_{FH}$ responses. Live bacteria purified bacterial RNA or synthetic agonists like CL075 equally promote $T_{FH}$ responses, which are not induced by other TLR ligands or inactivated bacteria (Fig. 22-25). These results open interesting possibilities for the use of synthetic or natural TLR8 ligands as vaccine adjuvants to supplement present-day vaccine preparations in the clinical practice as well as in the farming industry. Recently developed protein-based or subunit vaccines offer a safer alternative to live-attenuated vaccines, but, as mentioned, they often display markedly weaker immunogenicity and long-term protection. Adding adjuvants that augment immunogenicity could potentially combine the potency of live attenuated vaccines with the safety of inactivated or recombinant ones and is, therefore, highly desirable. Due to the vital role of $T_{FH}$ cells in promoting the germinal centre reactions, high affinity antibody production, and in fuelling the establishment of an immunological memory, aiding their development via adjuvants has become a strong focus of active research[187].

Several TLR ligands, as mentioned, are already used, or are currently under clinical evaluation, as vaccine adjuvants[213] with the aim of bridging the innate immune recognition to a potent activation of the adaptive responses. In particular, TLR7/8 ligands in the form of synthetic small molecule imidazoquinolines such as R-848 (resiquimod), R-837 (imiquimod), CL075 (3M002) or the highly TLR8-specific VTX-294 (and also other synthetic derivates[255]) are promising candidates as both anti-tumour treatments[256] and vaccine adjuvants, due to their potent effect of activating monocytes, Mo-DC and DC, inducing costimulatory molecules and production of cytokines including IL-1β, IL-12 and TNF[257-259]. TLR8 agonists have also shown effect on T cell responses and antibody secretion. In mice, whole-cell, heat-killed *Leishmania major*, when combined with subcutaneously injected R848, favoured a protective $T_H1$ response, thus improving vaccine efficacy[260]. Recent studies in particular have focused on the application of these agonists as adjuvants for vaccines against viral and bacterial diseases in adults and in new-borns (whose APCs express TLR8 at levels equivalent to adults)[261, 262]. Nanoparticles containing TLR8 agonist were found to induce responses similar to those induced by a live attenuate BCG

vaccination (but distinct from that of alum-adjuvanted subunit vaccines) including maturation of DC and production of IL-12p70[261]. The particles also affected the ensuing adaptive immune response and, when co-loaded with a mycobacterial antigen, promoted the generation specific $CD4^+$ T cells in TLR8 humanized mice. In a similar approach *poly(lactic-co-glycolic) acid* (PLGA) nanoparticles containing R848 and the TLR4 ligand MPLA, where shown to promote higher antibody titers where administered in combination with 2009 pandemic H1N1 influenza whole inactivated virus[263]. Supplementation of alum-adjuvanted pneumococcal glycoconjugate vaccine (PCV13 or Prevnar 13) with a TLR8 ligand showed increased circulating IgG responses in new-born rhesus macaques[264]. In the same model, the HIV Gag protein conjugated with a TLR7/8 ligand was found to promote robust $T_H1$ and $CD8^+$ T cell responses[262]. Given the role we have uncovered for TLR8 in the recognition of live bacteria and its impact on the vaccine response, an appealing clinical approach for the development of an efficient vaccine platform, would be the encapsulation of the purified microbial RNA into nanoparticles to "mimic" the *vita*-PAMP content of a viable bacteria.

While commonly licensed live attenuated vaccines are diverse in their composition and as such are likely to activate several complementary pathways[265], we propose innate immune

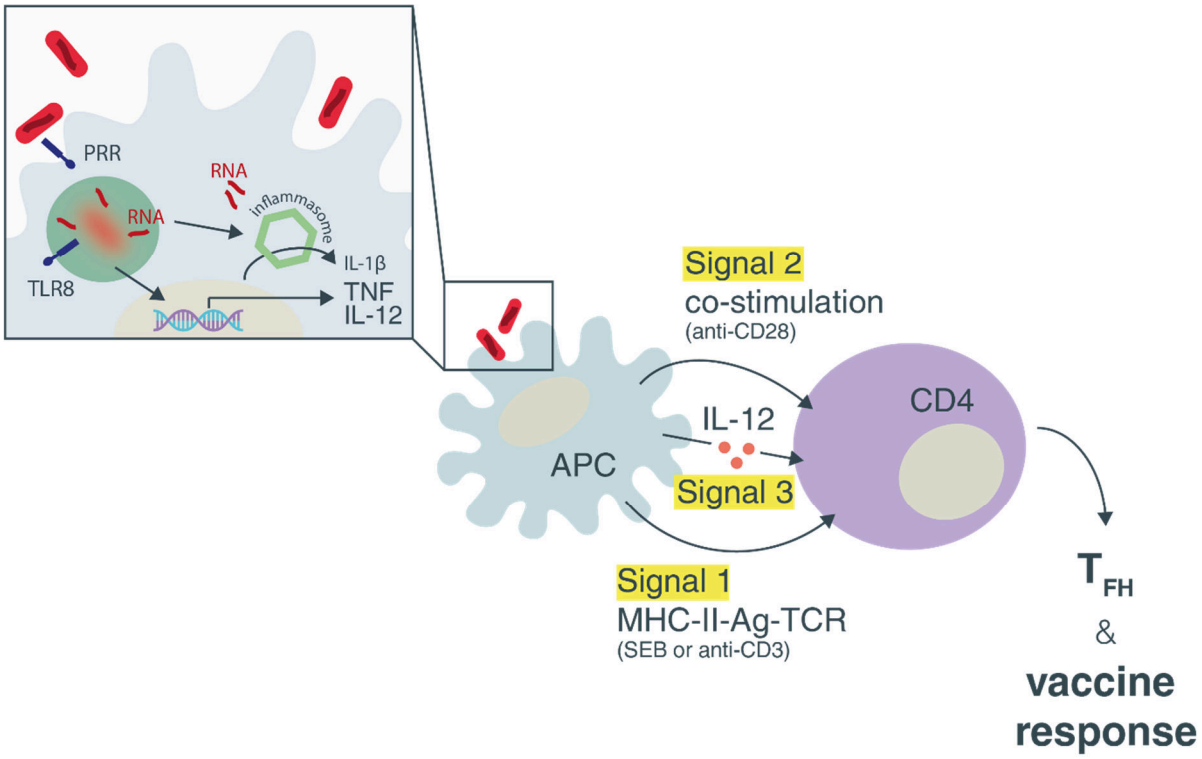


***Figure 38. Graphical depiction of the original findings reported in the present study***. Recognition of bacterial viability (and its signature bacterial RNA) in human APCs (magnification), mediated by endosomal TLR8, induced proinflammatory cytokines TNF and IL-12. The secreted cytokines (Signal 3), together with the MHCII-Ag-TCR immunological synapses (Signal 1) and the expression of surface activation markers (Signal 2) efficiently promotes the differentiation of naïve $CD4^+$ T cells into effector $T_{FH}$ cells with important implications for an effective response to vaccines.

recognition of microbial viability and subsequent elicitation of T cell-dependent antibody responses as a unifying *leitmotif* in the immune responses to live attenuated vaccines. In this light, understanding the elaborate processes behind the control of germinal center formation and the generation of affinity-matured, long lived memory and antibody producing cells is of central interest to guide the rational design of efficacious modern subunit vaccines.

## 4.5 Conclusion

In conclusion, the present study shows how the recognition of bacterial RNA, a molecular signature of microbial viability, a so-called *vita*-PAMP constitutes a powerful stimulus for $T_{FH}$ differentiation. Its recognition mediated by TLR8 after phagocytosis of live bacteria by APCs is an important immune checkpoint which promotes proper immune responses while, at the same time facilitates an accurate risk assessment limiting the risk of uncontrolled and deleterious[149, 266] $T_{FH}$ activation (***Fig. 38***) The identification of TLR8 as the first *vita*-PAMPs sensor in human and swine and master regulator of preferential $T_{FH}$ differentiation makes it an ideal candidate for the rational design of $T_{FH}$-targeted vaccine adjuvants against a broad range of infectious and non-infectious diseases.

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# Declaration of independent work

I, Matteo Ugolini, hereby declare that the submitted dissertation titled *"Recognition of microbial viability via TLR8 promotes innate and adaptive immunity"*, was written independently and without external help, and that I have completed the thesis autonomously using only the aids and tools specified.

I expressly declare that all sources used in the abovementioned work have been properly referenced as such. In particular, I declare that I have, without exception, stated the source for any statement.

I am aware that violations against the principles of academic independence are considered deception and are punished accordingly.

Date Signature